Cell-cell communication in *Streptococcus mutans* – From a global view to the single cell perspective

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2. Referent: Privatdozent Dr.-Ing. Max Schobert
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Lemme A, Sztajer H, Wagner-Döbler I. Characterization of *mleR*, a positive regulator of malolactic fermentation and part of the acid tolerance response in *Streptococcus mutans*. BMC Microbiol. 2010 Feb 23;10:58.

Kunze B, Reck M, Dötsch A, **Lemme A**, Schummer D, Irschik H, Steinmetz H, Wagner-Döbler I. Damage of *Streptococcus mutans* biofilms by carolacton, a secondary metabolite from the myxobacterium *Sorangium cellulosum*. BMC Microbiol. 2010 Jul 26;10:199.

Vilchez R, **Lemme A**, Ballhausen B, Thiel V, Schulz S, Jansen R, Sztajer H, Wagner-Döbler *Streptococcus mutans* inhibits *Candida albicans* hyphal formation by the fatty acid signaling molecule trans-2-decenoic acid (SDSF). ChemBioChem, 2010, 11, 1552-1562.

Sztajer H, **Lemme A**, Vilchez R, Schulz S, Geffers R, Yip CY, Levesque CM, Cvitkovitch DG, Wagner-Döbler I. Autoinducer-2-regulated genes in *Streptococcus mutans* UA159 and global metabolic effect of the *luxS* mutation. J Bacteriol. 2008 Jan;190(1):401-15. Epub 2007 Nov 2.

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Tagungsbeiträge

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A. Lemme, R. Vilchez, H. Sztajer, I. Wagner-Döbler.
Identifying the AI-2 controlled regulatory network in *Streptococcus mutans*. Annual Meeting Joint Annual Conference of the Association for General and Applied Microbiology. March 9-11, 2008. Frankfurt am Main, Germany

H. Sztajer, **A. Lemme**, R. Vilchez, S. Schulz, R. Geffers, C. Yip, C. M. Levesque, D. Cvitkovitch, I. Wagner-Döbler. The regulation of *Streptococcus mutans* transcriptome by autoinducer-2 and global metabolic effect of the *luxS* mutation. Cell-Cell Communication in Bacteria – ASM Conferences. October 7-10, 2007. Austin, Texas.

H. Sztajer, **A. Lemme**, R. Vilchez, M. Tosiek, I. N. Arthur, R. Geffers, I. Wagner-Dobler. Tracing the AI-2 activity in *Streptococcus mutans* UA159. Streptococcal Genetics – ASM Conferences. June 18-21, 2006. Saint-Malo, France.

Contents

Abbreviations

Aad9	Spectinomycin cassette
AMC	Activated methyl cycle
AB	Autoinducer bioassay medium
AHL	Acylated homoserine lactone
AI	Autoinducer
AI-2	Autoinducer-2
Amp	Ampicillin
APS	Ammonium persulfate
ATR	Acid tolerance response
BHIS	Brain Heart Infusion medium + sucrose
BMS	Biofilm forming media + sucrose
CAI	*Vibrio cholorae* autoinducer
CSP	Competence stimulating peptide
DPD	4,5-dihydroxypentane-2,3-dione
EMSA	Electrophoretic mobility shift assay
EPS	Exopolysaccharide
Erm	Erythromycin
FTF	Fructosyltransferase
GFP	Green fluorescent protein
Gm	Gentamycin
GTF	Glucosyl transferase
HAI	*Vibrio harveyi* autoinducer
HK	Histidine kinase of a two component system
HSL	Homoserine lactone
Km	Kanamycin
LB	Luria-Bertani medium
Luc	Gene of the firefly luciferase
MCS	Multiple cloning site
MLF	Malolactic fermentation
MS	Mutans streptococci
OD	Optical density
PBS	Phosphate saline buffer
PI	Propodium iodide
QS	Quorum-sensing
RR	Response regulator of a two component system
RT	Room temperature

SAH	*S*-adenosyl-homocysteine
SAM	*S*-adenosyl methionine
Sp	Spectinomycin
SRH	*S*-ribosyl-homocysteine
Tc	Tetracycline
TCS	Two component system
TEMED	N,N,N′,N′-Tetramethylethan-1,2-diamin
THBY	Todd Hewitt Broth medium + Yeast extract
tt	Terminator
wt	Wild-type
XIP	Sigma factor X inducible peptide

1 Chapter I - General introduction

1.1 Bacterial communication using autoinducers – interconnection between cell density, diffusion rate and spatial distribution

Quorum sensing

Over the last decades, the notion that bacteria act as selfish individuals has been replaced with the finding that bacterial populations can change collectively their behaviour to cope with changing environmental conditions. Pivotal for such a coordinated behaviour is the secretion and detection of small diffusing compounds with concomitant changes in gene expression. The original definition of quorum-sensing (QS) was dependent on an increase in the bacterial cell density, exceeding a threshold (quorum), that is coinciding with a certain amount of signalling molecules (autoinducers, AI; pheromones), needed to activate a signal transduction cascade, enabling the cooperative behaviour (Fuqua et al. 1994).

Diffusion sensing

Detection of autoinducers that were before released into the environment is a common mechanism for all QS systems. However, the definition of being a cell density dependent process was founded on clonal laboratory cultures and did not consider the *in-vivo* situation of a bacterial cell – living in a complex community and being exposed to permanent fluctuations of its environment. Redfield proposed a different interpretation for the release of autoinducer molecules into the environment, termed diffusion sensing (Redfield 2002).
In that view, autoinducer molecules are released to determine the diffusion rate in the immediate environment of a cell, rather detecting the cell density. Bacteria cannot use phagocytosis for the uptake of macromolecules, consequently they rely on the secretion of degradative enzymes to break down those molecules into subunits that can then be internalised. Connecting the diffusion rate of the immediate environment to the production of secreted substances, like exoenzymes, bacteriocins or siderophores, would prevent wasteful synthesis of these extracellular molecules. Thus, the relatively small metabolic burden for the production of autoinducers can reduce the overall metabolic costs of a single cell. This idea is supported by the fact that autoinducer regulated genes most commonly encode extracellular products.

Efficiency sensing

The concept of using autoinducers to sense the cell density to initiate a coordinated group behaviour has several drawbacks since it does not consider that the AI concentration is affected by diffusion and advection (mass transfer), spatial distribution of the cells, degradation and the production of the same AIs by other bacteria. Although the problem of mass transfer was solved by the idea of diffusion sensing (Redfield 2002), it does not account for the spatial distribution of cells in their habitat, as it was based on the view that individual bacteria sense their immediate environment. Estimation of the cell density, mass transfer and spatial distribution, to estimate the efficiency of producing extracellular effectors, was unified in the idea of efficiency sensing and was proposed by Hense *et al*. (Hense et al. 2007). The authors propose that bacterial communication can only assess the combination of cell density, spatial distribution and limitation to AI mass transfer. It conveys the idea that low cost AI molecules are secreted into the environment as a proxy for testing the efficiency of producing costlier diffusible extracellular effector molecules. Both, AIs and effectors are basically subjected to the same environmental influences, thus the estimated AI concentration will predict the expected presence of effectors, including the effect of cell density or mass transfer limitations. Moreover, the key determinants for the AI concentration cell density, mass transfer properties and spatial distribution are not coupled and can vary independently. Cooperative production of effectors is not always the case since individual cells can produce effectors by themselves. However, a few cells grown in close proximity in a microcolony will reach sooner a certain threshold than many single cells being more apart from each other. This effect is even more emphasized considering the usual positive feedback of AI production. Cell-cell communication in a microcolony is even more favoured due to a decreased possibility of AI interference or degradation since cells in a microcolony originated in most cases from the same species. In summary, this leads to the conclusion that cells cannot distinguish between cell density, mass transfer properties or spatial distribution and that they sense a combination of all factors. It unifies the concepts regarding what the cells sense (autoinducers), why they sense it (to estimate the efficiency of producing extracellular effectors) and that both individual cells and a group of cells will have most benefit from an optimized use of autoinducers (Hense et al. 2007).

Accordingly, coordinated group behaviour is not always coupled to the number of individual cells of a population but will depend on the relative amount of autoinducer molecules which is determined by the rate of synthesis, loss and degradation. However, since the term quorum sensing is commonly accepted to describe signalling or cell-cell communication in bacteria it will be used throughout the thesis.

1.2 Prokaryotic quorum sensing systems

Bacteria secrete a vast number of molecules into the environment, but what defines a molecule to be a quorum-sensing molecule? According to Winzer *et al*. (Winzer et al. 2002) a QS signal molecule has to

(i) accumulate in the extracellular milieu during a specific growth stage or under certain physiological conditions, or in response to specific environmental changes,
(ii) has to be recognized by a specific cell surface or cytoplasmic bacterial receptor,
(iii) has to have the ability to induce a cellular response that extends beyond the physiological changes required to metabolise or detoxify the molecule.

Several molecules belonging to different distinct chemical classes have been identified including e.g. the *N*-acylhomoserine lactones (AHLs), 2-alkyl-4-quinolones, and long fatty acid derivates, which are present in Gram-negative bacteria. Gram-positive bacteria predominantly utilize small posttranslationally modified peptides. Molecules shared by both groups are e.g. the autoinducer-2 furanone derivates or γ-butyrolactones.

1.2.1 Acylhomoserine lactone mediated quorum sensing

Acylhomoserine lactone mediated quorum-sensing is widespread in Gram-negative bacteria and is dependent on members of the LuxI family of AHL synthases and the cognate cytoplasmic receptor proteins, belonging to the LuxR family of transcriptional regulators. Many studies revealed that the regulation is more complex than initially thought, since the *luxI* and *luxR* genes are regulated by a number of transcriptional and post-transcriptional regulators responding to environmental conditions. Thus, embedding QS in a global regulatory network that adjusts AHL production and coordinated gene expression according to various environmental factors (Boyer and Wisniewski-Dye 2009). AHL molecules consist of a homoserine lactone (HSL) ring that is *N*-acylated with an acyl-chain of variable length, saturation level and oxidation state. Recently Schaefer *et al*. discovered a novel homoserine lactone derivate in *Rhodopseudomonas palustris*, that substitutes the acyl side chain with *p*-coumaroyl, and is presumably involved in the regulation of the chemotaxis of *R. palustris* (Schaefer et al. 2008). Until then, only *N*-acylated-HSL molecules had been identified in Proteobacteria. However, Schaefer *et al*. reported that the newly discovered *N*-(*p*-coumaroyl)-HSL is also produced by other bacteria (Schaefer et al. 2008) and thus extend the possibility of HSL quorum sensing to mediate interspecific signalling. Figure 1 shows the structure of *N*-

(*p*-coumaroyl)-HSL of *R. palustris* and the first discovered AHL, *N*-(3-oxohexanoyl)-L-HSL, that regulates luminescence in the marine bacterium *Vibrio fisheri* (Fuqua et al. 1994).

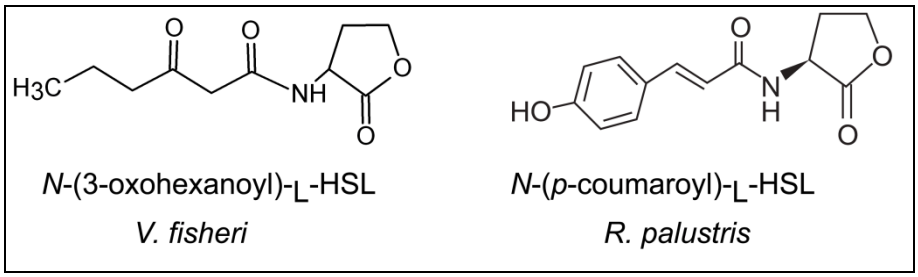

Figure 1. Structure of two homoserine lactone signalling molecules.
The *V. fisheri* HSL containing an acyl side chain and the HSL from *R. palustris* comprising an aroyl moiety were chosen as representatives for homoserine lactone signalling molecules.

1.2.2 Diffusible signal factor signalling

The first diffusible signal factor (DSF) was chemically characterized as *cis*-11-methyl-2-dodecenoic acid (Wang et al. 2004). It is synthesized by the plant pathogen *Xanthomonas campestris* and is involved in the regulation of extracellular enzymes, biofilm dispersal or toxin resistance (Dow and Daniels 1994, Dow et al. 2003, He et al. 2006). The *rpfCFG* genes have been shown to be required for the synthesis, detection and signal transduction of DSF molecules. RpfG possess also phosphodiesterase activity cleaving cyclic diguanosine monophosphate (c-di-GMP) and thus connects intercellular with intracellular signalling (Ryan et al. 2006). Identical or similar molecules have been identified in other species that are associated with plants or soil, i.e. *Stenotrophomonas maltophilia* or *Burkholderia spp.*. Previously, production of a DSF molecule (*cis*-2-decenoic acid) has been reported in the human pathogen *Pseudomonas aeruginosa*. The molecule was shown to be involved in the dispersion of biofilms not only in *P. aeruginosa* but also in other Gram-negative and Gram-positive bacteria and in the opportunistic human pathogen *Candida albicans* (Davies and Marques 2009). Recently, Vilchez *et al*. discovered for the first time a DSF molecule produced by a Gram-positive bacterium, *Streptococcus mutans*, and elucidated its structure to be *trans*-2-decenoic acid. The molecule was shown to suppress the transition from yeast to hyphal morphology in *Candida albicans* at concentrations occuring in spent culture media of *S. mutans* and thus represents a interkingdom signalling molecule of two inhabitants of the oral cavity (Vilchez et al. 2010).

1.2.3 Autoinducer-2 mediated quorum sensing

Autoinducer-2 (AI-2) is a term that describes various furanone derivates that arise from the molecule (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) through spontaneous rearrangements and incorporation of borate. The LuxS (lux-luminescence, S-synthase) enzyme is required for the production of DPD and is integrated in the activated methyl cycle (AMC) of the cell. Autoinducer-2 signalling was first discovered in the marine bacterium *Vibrio harveyi*, where it is involved amongst other autoinducers in the regulation of luminescence (Bassler et al. 1993, Bassler et al. 1994). The widespread distribution of the *luxS* gene suggested that AI-2 molecules could mediate interspecies communication (Bassler et al. 1997, Sun et al. 2004) and tempted many researchers to study the role of AI-2 in various microorganisms. However, analysing the role of AI-2 in the past was very challenging since chemical autoinducer-2 was not available and deletion of *luxS* is always associated with a disruption of the AMC (see below). Thus, it was not possible to assign observed phenotypes to either AI-2 signalling or to the disrupted metabolism of the AMC.

Production of autoinducer-2

The AI-2 producing enzyme LuxS is incorporated in the AMC (Figure 2). This cycle is present in each free-living organism and fulfils a central role in the metabolism of the cell, since it detoxifies SAH and the homocysteine moiety of SAH is reused for methionine synthesis. Furthermore, all the enzymes within the AMC are well-conserved, and thus it is likely that they serve the same function in each bacterium (Winzer et al. 2002).

During various methyl transferase reactions *S*-adenosylmethionine (SAM) is converted to *S*-adenosylhomocysteine (SAH), which is an potent feedback inhibitor of SAM-dependent methylations, due to its structural similarities, and has to be recycled (Winzer et al. 2002).

Recycling of SAH can be achieved using a one step mechanism (pathway I in figure 2) or a two step mechanism involving the LuxS enzyme (pathway II in figure 2). The one step conversion from SAH to homocysteine and adenosine is preferentially used by all eukaryotes, and archaebacteria, and some eubacteria (Sun et al. 2004). Detoxification of SAH using the SAH-hydrolase (SahH) by-passes the production of DPD and thus might represent a strategy to complement a deleted *luxS* gene and to study the role of exogenous added DPD.

The two-step pathway is exclusively used by bacteria. In the first step the toxic SAH molecule is converted by the Pfs enzyme and yields *S*-ribosylhomocysteine (SRH) and adenine. In the second step, SRH is cleaved by the LuxS enzyme to homocysteine and (*S*)-4,5-dihydroxy-2,3-

pentanedione (DPD), which then spontaneously rearranges to various furanone derivates, which are collectively termed AI-2 (Chen et al. 2002, Miller et al. 2004, Schauder et al. 2001).

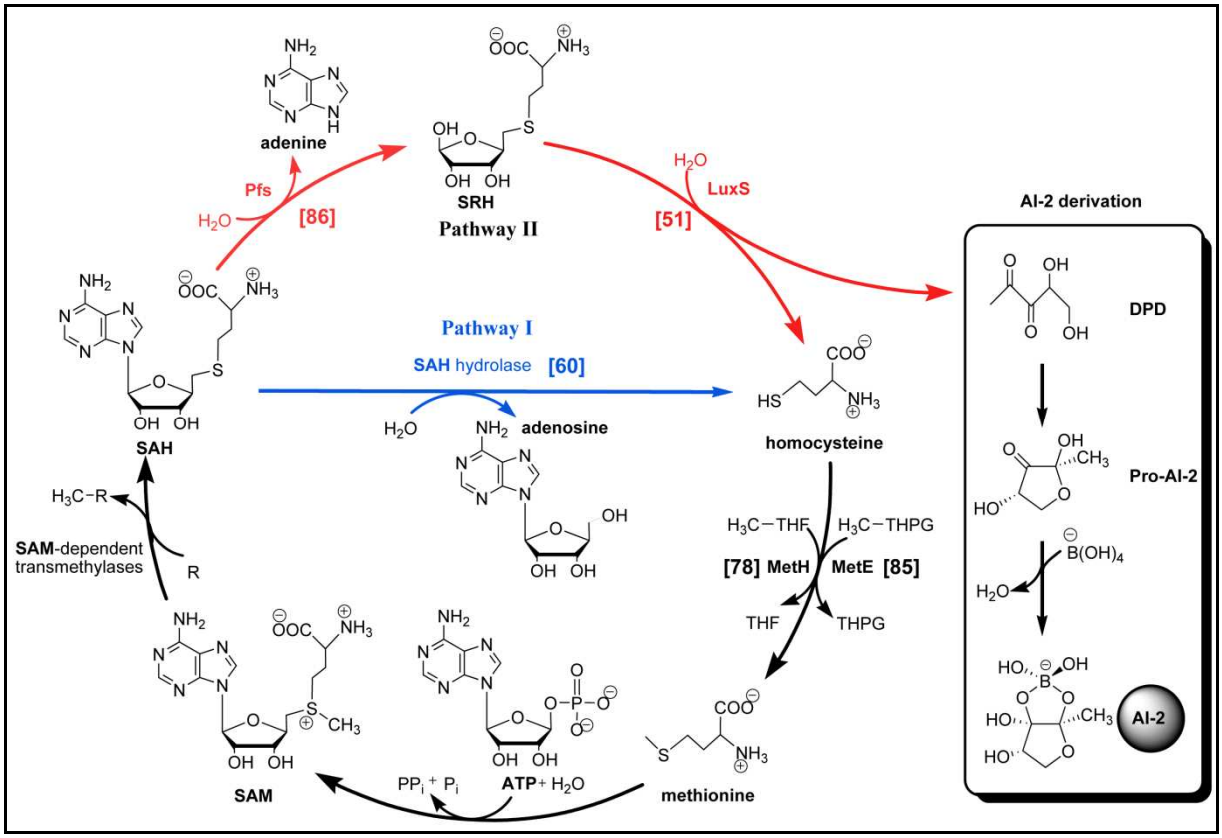

Figure 2. Both alternative forms of the activated methyl cycle modified after Sun *et al.*.
Enzymes involved in the detoxification of SAH and synthesis of AI-2. Abbreviations: SAM, S-adenosyl-methionine; SAH, S-adenosyl-homocysteine; SRH, S-ribosyl-homocysteine; DPD, 4,5-dihydroxyl-2,3-pentanedione; Pro-AI-2, AI-2 precursor; AI-2, autoinducer 2. The numbers in brackets show the numbers of analyzed organisms that have that enzyme (reciprocal best hit) (Sun et al. 2004).

Epimeric forms of autoinducer-2 – Chemical interconversions of DPD

DPD is produced by the LuxS enzyme and in aqueous environments it cyclizes spontaneously to form two epimeric furanones, *S*-DHMF and *R*-DHMF (Figure 3). Both molecules become hydrated and form *S*-THMF and *R*-THMF. The stereoisomers are in equilibrium and can interconvert through the open form of DPD. It was found that the *R*-THMF furanone binds to the LsrB receptor of the enteric bacteria *Salmonella typhimurium* and *Escherichia coli*. High concentrations of borate favour ester formation yielding *S*-THMF-borate. Since borate is abundant in marine environments (~ 0.4 mM) this form is recognized by the LuxP receptor protein of the marine bacterium *V. harveyi*.

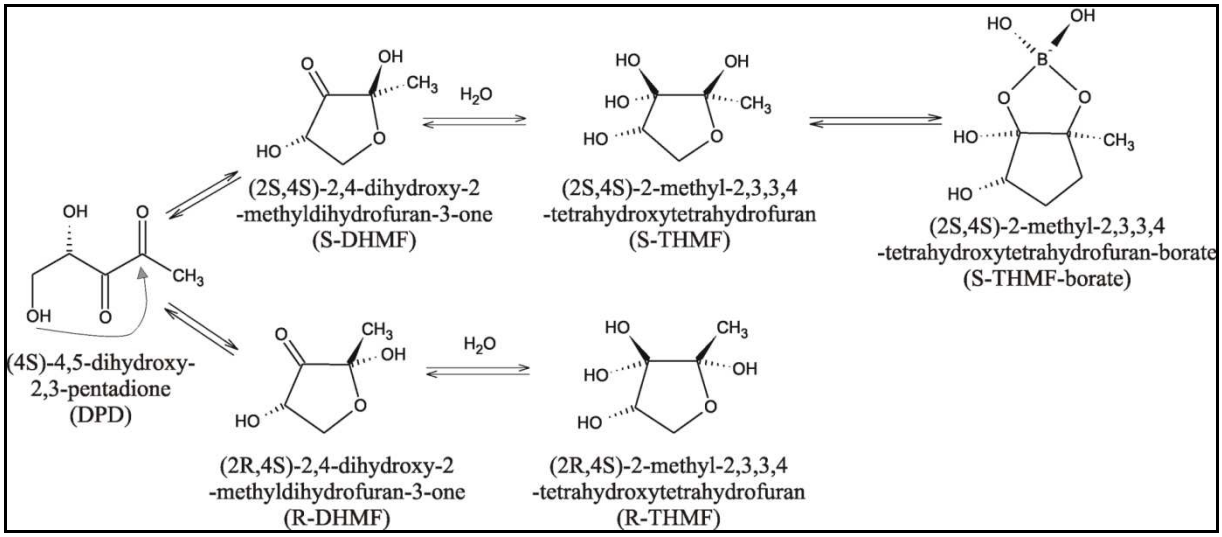

Figure 3. Chemical interconversions of DPD that lead to two distinct signalling molecules.
The linear form of DPD spontaneously cyclizes into two epimeric furanones which become hydrated. In the presence of borate the stereoisomer *S*-THMF forms a borate diester and is sensed by *Vibrio sp*.. The other stereoisomer *R*-THMF is recognized by the LsrB receptor from *S. typhimurium* and *E. coli*. The image is adapted from Miller et al. (Miller et al. 2004).

The role of AI-2 in *Vibrio sp.*

Autoinducer-2 mediated quorum sensing is best studied in *Vibrio sp.* and is described below for *V. harveyi* as a representative of the genus Vibrio and of Gram-negative proteobacteria, respectively.

In *Vibrio sp*. autoinducer-2 is integrated in a multi input quorum-sensing system that controls the expression of genes that are involved in the regulation of bioluminescence and various other traits (figure 4). Three different signal molecules, HAI-1 [AHL, synthesized by LuxM], CAI-1 [(*S*)-3-hydroxytridecan-4-one, synthesized by CqsA] and AI-2 (borate diester of DPD, synthesized by LuxS) are detected by distinct membrane bound autophosphorylating histidine sensor kinase proteins (Kelly et al. 2009, Tu and Bassler 2007). LuxN detects HAI-1, CqsS senses CAI-1 and LuxPQ recognizes AI-2. All three sensors can act as kinases or phosphatases, depending on the concentration of their cognate signal molecules. At low cell density (i.e., when AI concentration is low) the histidine-containing phosphotransfer protein LuxU becomes phosphorylated. The phosphates are transferred to LuxO. Together with δ^{54}, LuxO~P activates the expression of five small regulatory RNAs (sRNAs), named *qrr*. In conjunction with the RNA chaperone Hfq, the *qrr* sRNAs destabilizes the mRNA of the master regulator LuxR. As a consequence LuxR protein levels are kept at a low level when the concentration of autoinducers is low.

At high cell densities (i.e., when AI concentration is high) LuxU and LuxO become dephosporylated and expression of the *qrr* sRNAs is decreased. The protein levels of LuxR

increase and the quorum response can be activated. Due to the parallel input of three signal molecules on three different receptor molecules and the redundancy of *qrr* sRNAs the protein level of LuxR can be modulated very precisely. As a consequence a gradient of LuxR protein molecules induces a gradient of expression of quorum sensing target genes (Tu and Bassler 2007). This clearly shows that the use of autoinducers is not coupled directly to a quorum, resulting in an all or none-induction after a certain threshold is exceeded, but that different autoinducers are used to fine-tune the global expression of a population.

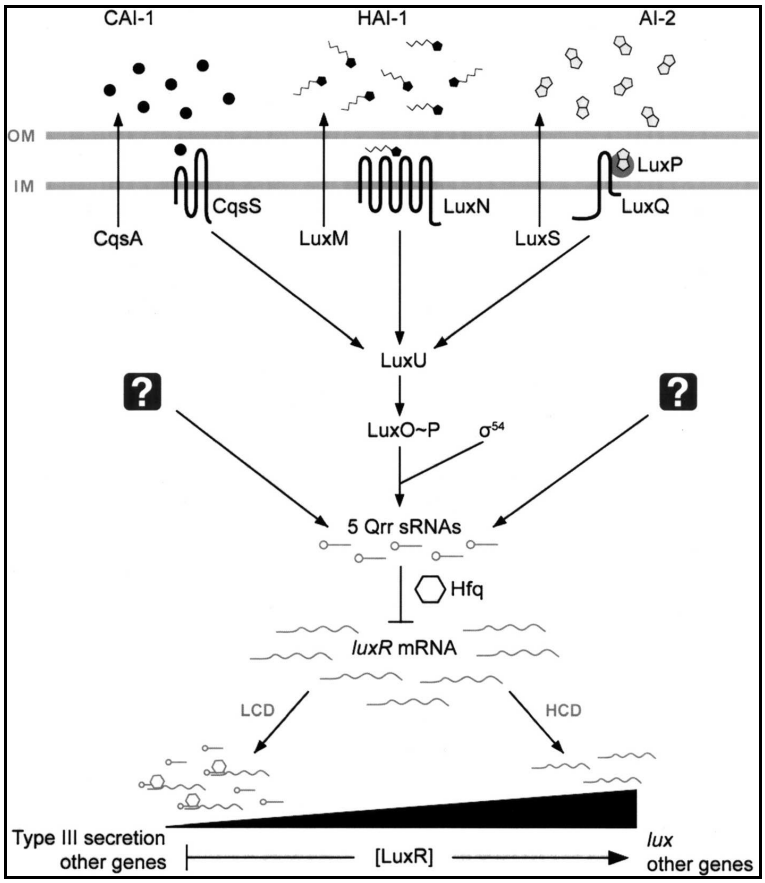

Figure 4. Model of the *V. harveyi* quorum sensing system.
Three sensory system act in concert to modulate the gene expression of the master regulator LuxR. The concentration of all three signal molecules and the sigma factor σ54 are involved in the expression of five small regulatory RNAs, which destabilize luxR mRNA and thus fine tune its expression. Different levels of concentrations of LuxR control the expression of various genes. Adapted from Tu and Bassler (Tu and Bassler 2007).

Staphylococcus aureus is a major nosocomial pathogen with the ability to cause a variety of infectious diseases and is chosen here as a member of the Gram-positive bacteria for AI-2 signalling.

Its pathogenic determinants are controlled by a wide range of regulatory elements, including the peptide autoinducer Agr system. However, a recent study demonstrated that *S. aureus* uses AI-2 to regulate capsular polysaccharide (CP) production. According to the definition of Winzer *et al*. (Winzer et al. 2002) what defines a molecule to be a signalling molecule. The effect of AI-2 on CP production in *S. aureus* has to be assigned to a signalling effect, since AI-2 is secreted into the environment, is detected by a two component system and activates the expression of genes other than those required for its synthesis or degradation. CP is an important cell wall component that can interact with the host immune system during the invasive process, allowing *S. aureus* to resist uptake and killing by phagocytes (Zhao et al. 2010). Zhao *et al*. showed that DPD and the two component system KdpDE are involved in the regulation of the *cap* genes, which are responsible for CP synthesis. Interestingly, DPD was required in a concentration range of 39 nM – 3.9 µM to restore elevated *cap* expression in the *luxS* mutant back to wildtype levels. Outside of this concentration range no effect was observed (Zhao et al. 2010). Effects of AI-2 in a very narrow concentration range were also seen in other bacteria, demonstrating that AI-2 complementation experiments can be very challenging (Ahmed et al. 2009, Rickard et al. 2006). Contradictory to Gram-negative bacteria, detailed mechanisms of regulatory cascades in Gram-positive bacteria are currently not known.

Various phenotypes in several different bacteria have been reported to be related to a deletion of the *luxS* gene. Most of them were associated with a deficiency in biofilm formation, but also defects in other important virulence traits have been reported. Only a few studies have been carried out that were aimed to determine the role of AI-2. Table 1 lists studies that used chemically or enzymatically synthesized AI-2 or purified autoinducer-2 from bacterial supernatants, respectively, to restore phenotypes caused by the deletion of *luxS*. Studies that investigated only *luxS* deletion mutants and its complementation with spent culture supernatants or *luxS* expression in *trans* are not included. Since supplementation with pure AI-2 resulted in some cases in a (partial) complementation of the phenotypes caused by the lack of *luxS* and since transporter and receptor proteins other than LuxP and LsrBR have been identified indicate that AI-2 indeed has the potential to serve as an interspecies signalling molecule.

The role of AI-2 in *Streptococcus mutans*

In the study of Sztajer *et al*. a complementation experiment with chemically synthesized AI-2 and a *luxS* mutant was performed. Microarray analysis revealed that 59 genes were differentially regulated in the presence of AI-2. Among these genes, putative regulatory elements as the delta subunit of the RNA polymerase, RpoE, or the transcriptional regulator, MleR, have been found. However, no relationship between AI-2 and these two genes could subsequently be confirmed (unpublished results).

Table 1. Influence of *luxS* deletion and AI-2 complementation in diverse bacteria.

Strain	Phenotype *luxS* mutant	Complementation / autoinducer-2 preparation	Reference
Aggregatibacter actinomycetemcomitans	- Reduced biofilm formation - Reduced iron uptake Two importers (LsrB and RbsB) and a TCS (QseBC) are known to be involved	Partially purified AI-2 from culture supernatants restored the phenotypes.	(Shao and Demuth 2010)
Escherichia coli	- Reduced biofilm formation - Reduced motility through altered flagallar synthesis The importer LsrB, the TCS QseBC and the regulator MqsE are known to be involved	Enzymatic synthesized AI-2 using LuxS/Pfs restored the phenotypes.	(Gonzalez Barrios et al. 2006)
Escherichia coli EHEC	Reduced expression of the LEE type III secretion system	Chemically synthesized AI-2. 10-250 µM did not restore the phenotype.	
Helicobacter pylori	- Reduced motility through altered flagellar synthesis	Commercial chemically synthesized AI-2. 150 µM AI-2 restored the phenotype.	(Shen et al. 2010)
Lactobacillus rhamnosus GG	- Reduced biofilm	Chemically synthesized AI-2. 1 nM – 100 µM did not restore the phenotype.	(Lebeer et al. 2007)
Listeria monocytogenes EGD-e	- Increased biofilm formation	Enzymatic synthesized AI-2 using LuxS/Pfs (50 µM) did not restore the phenotype.	(Challan et al. 2006)
Neisseria meningitidis	- Reduced virulence	Enzymatic synthesized AI-2 using LuxS/Pfs. Addition of this preparation to the *luxS* mutant revealed no alterations in the proteome.	(Schauder et al. 2005)
Salmonella typhimurium	- Reduced biofilm	□□□□□□ally synthesized AI-2. 72 µM did not restore the phenotype.	(De Keersmaecker

			et al. 2005)
Staphylococcus aureus	- Decreased capsular polysaccharide synthesis	Commercial chemically synthesized AI-2. 39-390 nM AI-2 restored the phenotype	(Zhao et al. 2010)
Streptococcus anginosus	- Higher susceptibility to antibiotics	Commercial chemically synthesized AI-2. 1.5-1.8 nM AI-2 restored the phenotype.	(Ahmed et al. 2007)
Streptococcus epidermidis	- Differential gene expression of metabolic and virulence associated genes (e.g. lipase, phenol-soluble modulins)	Enzymatically synthesized AI-2 using LuxS/Pfs restored the phenotypes.	(Li et al. 2008)
Streptococcus intermedius	- Increased biofilm formation in the presence of antibiotics	Commercial chemically synthesized AI-2. 0.8 nM AI-2 restored the phenotypes.	(Ahmed et al. 2008, Ahmed et al. 2009)
Streptococcus intermedius	- Decreased haemolytic activity	Commercial chemically synthesized AI-2. 0.4 - 32 nM AI-2 restored the phenotype.	(Pecharki et al. 2008)
Streptococcus mutans UA159	- Differential gene expression of diverse genes	Chemically synthesized AI-2. AI-2 was added twice at different time points to a concentration of each 75 µM and altered the expression of 59 genes.	(Sztajer et al. 2008)
Streptococcus oralis	- Reduced biofilm in co-culture with *Actinomyces naeslundii*	Chemically synthesized AI-2. 0.08-0.8 nM AI-2 restored the effects of *luxS* mutation in dual species biofilms but not in single species biofilms	(Rickard et al. 2006)

1.2.4 Peptide mediated quorum sensing

Gram-positive bacteria predominantly utilize small posttranslationally modified peptides for the regulation of quorum sensing. Amongst many other traits, genetic competence – the ability to uptake DNA from the environment and integrate it into the genome, is regulated by QS and is described below for the soil bacterium *Bacillus subtilis* and the human pathogen *Streptococcus pneumoniae*.

The competence system of *Bacillus subtilis*

Quorum sensing describes the coordinated behaviour of a bacterial population in response to signalling molecules. This is true for competence development in *B. subtilis* but instead of triggering a population wide response, only a subpopulation changes its gene expression and becomes competent. This intra-population diversity of a clonal population has been referred to as “bistability”, due to the dual stable pattern of gene expression in genetically identical cells (Dubnau and Losick 2006). According to Ferrell, two mechanisms exist to manifest bistability in a bacterial population (Ferrell, Jr. 2002). One is the principle mechanism of competence initiation in *B. subtilis* and is described below. It requires the presence of a positively auto regulated transcriptional activator, which responds to itself in a non-linear manner. Specifically, above a certain threshold of the regulator a hypersensitive change in gene expression is induced due to the activation of the positive auto feedback loop. Consequently, this results in high levels of the regulator, and its controlled genes will be activated (Avery 2005). Cells that do not exceed the threshold will remain inactive, resulting in segregation of the clonal population. In *B. subtilis*, ComK fulfils these criteria and induces the expression of all genes in the competence pathway in a subpopulation.

Competence development in *B. subtilis* is triggered at the onset of the stationary phase. Two peptides, that accumulate at high cell density or under conditions of starvation are involved in initiating competence, named ComX and CSF/Phr (competence sporulation factor) (Figure 5). ComX is recognized by the two component system ComAP and leads to its activation through phoshporyl transfer from ComP to ComA. ComA~P triggers expression of *comS*, a small ORF in the surfactin-production operon (*sfr*). CSF is a processed peptide, originating from the *phrC* gene. Upon its accumulation in the environment it is transported back into the cell, via the Opp oligopeptide permease, and blocks the action of RapC (response regulator aspartate phosphatase C). RapC dephosphorylates ComA, thus increased levels of CSF result in a higher amount of ComA~P and subsequent higher levels of ComS. ComS antagonizes with the ternary complex that the master regulator ComK forms with MecA and ClpC, thus

preventing degradation of ComK by ClpP. The positive autoregulatory properties of ComK are essential for competence development. Due to the stabilization of ComK by ComS, a positive auto feedback loop of ComK can be established. The intracellular concentration of ComK increases, sufficient to drive expression of all genes that are necessary for DNA uptake and processing (Dubnau and Losick 2006, Hamoen et al. 2003). Regulation of ComK is very complex, since at least three repressors, one activator and ComK itself act on the *comK* promoter. Noise, or random fluctuations, in the expression of ComK and ComS in individual cells determine which cells initiate the positive ComK feedback. Because a certain threshold concentration of ComK has to be exceeded in a certain time window during growth, only a defined number of cells initiate competence (Leisner et al. 2007, Leisner et al. 2008).

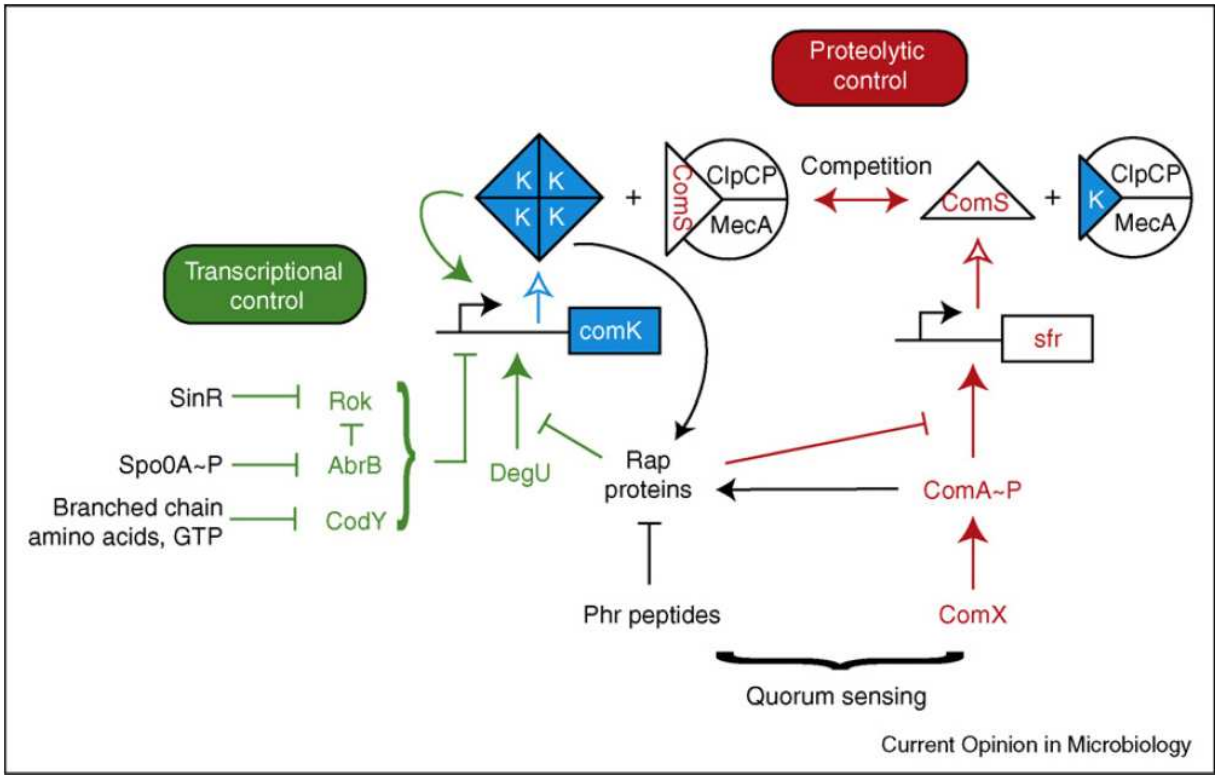

Figure 5. Simplified regulatory circuit controlling competence development in *B. subtilis*.
Transcriptional control of *comK* in green, proteolytic control of ComK in red. Filled arrows and T-bars indicate positive and negative regulation, respectively. Kinked arrows illustrate promoter regions, open arrows expression. K, ComK; ~P, phosphorylated (Leisner et al. 2008). Activation of the late competence genes (DNA uptake and recombination) by ComK is not shown.

The competence system of *Streptococcus pneumoniae*

Acquisition of DNA by natural transformation has been reported for members of the mitis and anginosus group of streptococci. The best characterized organism within these groups is *S.*

pneumoniae and is described below as paradigm, since the members of those groups share regulatory features required for initiation of competence, thus it is suggested that they regulate competence in a very similar way (Martin et al. 2006, Mashburn-Warren et al. 2010). Contradictory to *B. subtilis*, competence development in *S. pneumoniae* occurs population wide and a transformation rate of ~100% can be achieved under laboratory conditions (Claverys et al. 2007). During growth the gene product of *comC* is processed and released into the environment using the ComAB secretion apparatus (Figure 6). The mature peptide of ComC, named CSP (competence stimulating peptide) accumulates and above a certain threshold it is sensed by the membrane embedded histidine kinase ComD which activates its cognate response regulator ComE by phosphorylation. This signalling event occurs in the early to mid log phase but can be also triggered independently from cell density, by shifting the pH of the culture to alkaline conditions. Thus, CSP serves more as a stress signal rather than as an indicator of the cell density (Claverys et al. 2006).
However, phosphorylation of ComE leads to a positive autoregulation of the *comCDE* operon and induction of the so called early competence genes, including the *comAB* operon and the alternate sigma factor ComX. Expression of the late competence genes (DNA uptake and processing) is activated by ComX, since it directs the RNA polymerase to the late competence gene promoters. Upon CSP addition, maximal transcriptional activation of the early and late genes is achieved after 5-7 minutes and 10-12 minutes, respectively, afterwards levels of mRNA decay rapidly (Peterson et al. 2004, Rimini et al. 2000). The time window to obtain transformants is approximately 30 minutes post induction (Johnsborg and Havarstein 2009). Besides competence induction, other genes are simultaneously induced upon CSP addition. Among them are genes that trigger cells lysis and genes that confer immunity against cell lysis. These killing factors includes two murein hydrolases, LytA and CbpD, and a two component bacteriocin CibAB. Since killing and immunity factors (e.g. ComM) are under the control of the same regulators, competent cells are immune to lysis. Thus, induction of competence will result in lysis of non-competent siblings, a phenomenon referred to as fratricide (Claverys et al. 2007, Johnsborg and Havarstein 2009).

Leaving the competent state

In *S. pneumoniae* transcription of the genes, involved in the regulation of competence, is very short-lived and decay occurs within some minutes, thus a mechanism has to exist that control this strict regulation. It was shown that CSP activated expression of *comE* reached its maximum after 5 minutes but was almost undetectable 20 minutes post induction (Alloing et al. 1998), whereas the ComE protein was stable for at least 80 minutes. Since activation and de-activation of *comX* follows the pattern of *comE* with a few minutes delay, another regulatory element must exist that interferes with ComE mediated expression of *comX*. Turlan *et al*. identified such a regulator (termed SpxA1) and demonstrated that over-expression of

SpxA1 resulted in a repression of both early and late competence genes (Turlan et al. 2009). The regulator belongs to the family of Spx proteins, which is highly conserved in low-G+C content bacteria. Several studies demonstrated that Spx proteins participate in the general stress response of bacteria (Nakano et al. 2003, Kajfasz et al. 2010). Using *B. subtilis* as a model, Nakano and co-worker demonstrated that Spx proteins interact with the C-terminal domain of the alpha subunit (α-CTD) of the RNA polymerase (RNAP) (Nakano et al. 2003). The α-CTD mediates the contact of DNA-bound transcriptional regulators with the RNAP, resulting in an elevated transcription. The Spx protein of *B. subtilis* was shown to interfere with this interaction and thus inhibit transcription, despite the presence of a transcriptional regulator that would activate transcription (Nakano et al. 2003). Mutation of two residues of SpxA1 in *S. pneumoniae*, known to be involved in the interaction with the α-CTD of RNAP, significantly decreased the repressing effect of SpxA1 overexpression (Turlan et al. 2009), suggesting a similar mechanism as in *B. subtilis* for *S. pneumoniae* and presumably in all low-G+C content bacteria.

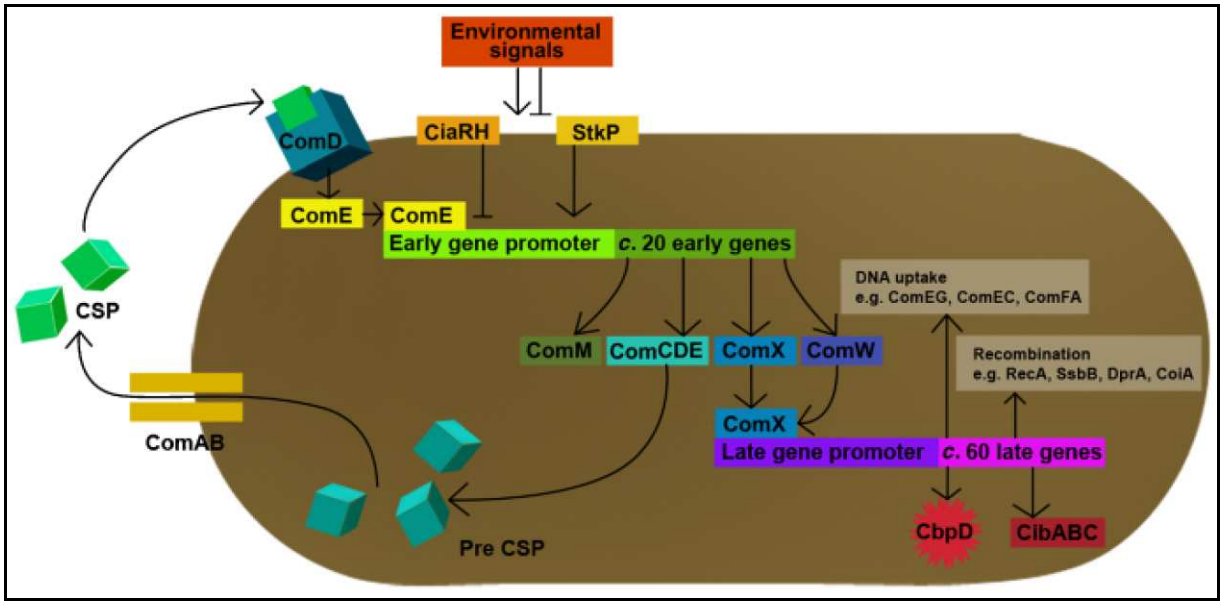

Figure 6. Schematic representation of competence regulation in *S. pneumoniae*.
The CSP precursor, which is encoded by the *comC* gene, is processed and secreted by the dedicated ComAB transporter, resulting in extracellular accumulation of mature CSP. Basal transcription of the *comCDE* operon is subjected to regulation by global regulators such as the serine/threonine protein kinase StkP and the CiaRH two-component system. Binding of CSP to its ComD receptor results in autophosphorylation of ComD and subsequent transfer of the phosphoryl group to the ComE response regulator. ComE then binds to and activates transcription from the various early gene promoters. ComE binding sets off increased transcription of the *comCDE* operon, leading to a boost in the production of CSP, ComD and phosphorylated ComE. This auto-induction loop ensures rapid accumulation of the alternative sigma factor ComX, ComW and the ComM fratricide immunity protein. ComW protects ComX from proteolytic cleavage and stimulates the latter protein to activate transcription of the late genes encoding the fratricide trigger factors CbpD and CibAB as well as the protein apparatus for DNA uptake and recombination. While *cibAB* is co transcribed with a cognate immunity gene (*cibC*), competent cells are protected from the CbpD murein hydrolase by the product of the early gene *comM* (Johnsborg and Havarstein 2009).

1.2.5 Quorum quenching

Quorum sensing is very often involved in the regulation of virulence factors or other traits helping bacteria to cope with its surrounding. Thus, the mechanism of QS represents an ideal target to compete with bacteria living in the same habitat or to interfere with bacterial pathogenicity and thus mechanisms that interefere with bacterial signalling can be found in bacteria and eukaryotes. A variety of bacteria (e.g. *Bacillus sp*.) is known to secrete so-called lactonases to compete with their AHL-producing neighbours, since these enzymes are capable of degrading AHLs by hydrolysing the lactone ring or by cleaving the acyl side chain (Zhang and Dong 2004).

Halogenated furanones represent a family of chemical molecules known to interfere with some QS systems of bacteria. These molecules are produced by the Australian macroalga *Delisea pulchra* and are active because they accelerate the turnover time of the bacterial LuxR-regulator (Manefield et al. 2002). Consequently, the amount of LuxR proteins within the bacterial cell is decreased and no quorum response can be activated even above the threshold of autoinducer molecules. Due to the production of furanones *D. pulchra* protects itself against the colonization of bacteria that use AHL dependent quorum sensing to establish their attachment to the algae. Moreover, synthetic furanones were shown to interfere with the quorum sensing signalling pathway in *V. harveyi* and some oral streptococci (Lonn-Stensrud et al. 2007).

Since the production of QS molecules is widespread in bacteria, it seems reasonable that antagonizing molecules will occur frequently in nature, too. Thus, the screening and identification of such compounds represents a good strategy to find new biologically active metabolites interfering with the pathogenicity of bacteria. One result of such a screening was the discovery that carolacton, a secondary metabolite isolated from the myxobacterium *Sorangium cellulosum* affected the biofilm formation of *Streptococcus mutans* (Jansen et al. 2010, Kunze et al. 2010). Carolacton was of particular interest because it showed a high antibacterial activity against biofilms of *S. mutans* but was inactive in standard bacterial growth inhibition tests of other Gram-positive and Gram-negative bacteria (Kunze et al. 2010). Kunze *et al*. reported that the antibacterial activity against biofilms of *S. mutans* was reduced in a mutant defective for ComD, the histidine kinase required to sense the competence stimulating peptide (CSP) of *S. mutans* (see below). Moreover, the expression of the alternate sigma factor ComX which is indirectly activated by CSP was significantly decreased upon addition of carolacton, suggesting that carolacton interferes with the quorum sensing network of *S. mutans* (Kunze et al. 2010).

1.3 The oral cavity and *Streptococcus mutans*

Streptococcus mutans is a Gram-positive bacterium that was isolated by J. K. Clark in 1924 from a carious lesion and cells were described to be small, chained coccobacilli. *S. mutans* belongs to the so-called mutans group of streptococci which has been divided into eight serotypes designated a-h due to differences of carbohydrates in the cell wall (Marsh and Martin 2000). Furthermore, DNA homology analysis classified mutans streptococci (MS) into seven distinct species – known as *S. mutans* (serotypes c, e and f), *S. sobrinus* (serotypes d and g), *S. cricetus* (serotype a), *S. ferus* (serotype c), *S. macacae* (serotype c) and *S. downei* (serotype h). Experiments in the 1960s presented evidence that these organisms, especially *S. mutans* and *S. sobrinus*, belong to the primary etiologic agents of human dental caries and exist almost exclusively in biofilms on tooth surfaces.

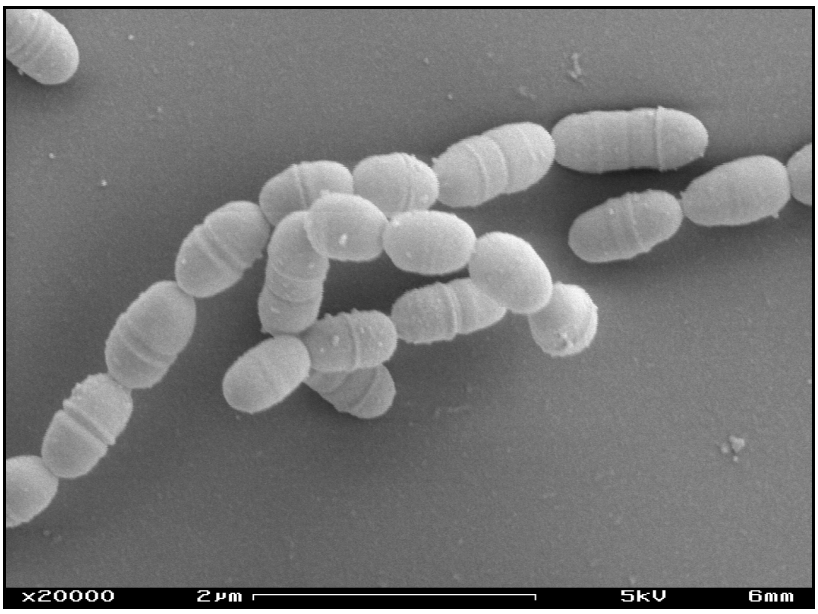

Figure 7. Scanning electron microscope image of *S. mutans* UA159.
The scanning electronic microscopic image shows *S. mutans* U159 wildtype cells grown in complex medium. With kind permission of Xiaoli Xue and Manfred Rohde. The scale bar is two micrometers in size.

1.3.1 Development of dental plaque biofilms

S. mutans is part of the very complex and diverse community of the oral microflora, which comprises more than 700 bacterial species (Aas et al. 2005, Faveri et al. 2008, Paster et al. 2006). The ability of bacteria in the oral cavity to adhere to (tooth) surfaces, mucosa,

epithelial cells and to each other allows the formation of a very complex multispecies biofilm. Surface attachment is crucial for the persistence in the oral cavity since non-adherent bacteria would be washed away by saliva. Moreover, biofilm formation increases the ability of the individual cells to withstand the harsh environmental conditions such as varying nutrient availability, different oxygen levels, pH changes and the exposure to detergents during tooth brushing (Carlsson 1997). The colonization of the human teeth (dental plaque) starts with the formation of the pellicle, a thin layer of host derived molecules that coat the tooth enamel surface within minutes after professional cleaning. The pellicle is a source of receptors that the early colonizers of the human teeth bind to and is comprised of mucins, agglutinins, proline-rich proteins, phosphate-rich proteins, and enzymes such as alpha-amylase (Kolenbrander et al. 2002).

Based on cultivation studies oral streptococci (e.g. *S. gordonii, S. mitis, S. sanguinus, S. anginosus, S. oralis S. salivarius, S. mutans* and *S. sobrinus*) belong to the early colonizers and constitute 60-90% of the bacteria that colonize the teeth within the first 4 h after professional cleaning (Nyvad and Kilian 1987). Attachment, growth and metabolism of the early colonizers provides suitable conditions and nascent surfaces for the attachment of other bacteria (late colonizers), resulting in an ordered pattern of colonization (microbial succession) and concomitant changes in the species diversity (Kolenbrander and London 1993, Socransky et al. 1998). Moreover, the metabolic activity of the early colonizers changes the environmental conditions or produces metabolites that are necessary for the colonization of late colonizers. A model representing the interaction during dental plaque development is shown in figure 8 (Kolenbrander et al. 2002). The overall abundance of mutans streptococci (e.g. *S. mutans* and *S. sobrinus*) in dental plaque is low until conditions are established that favour the growth of these acid-tolerating (cariogenic) bacteria (see below).

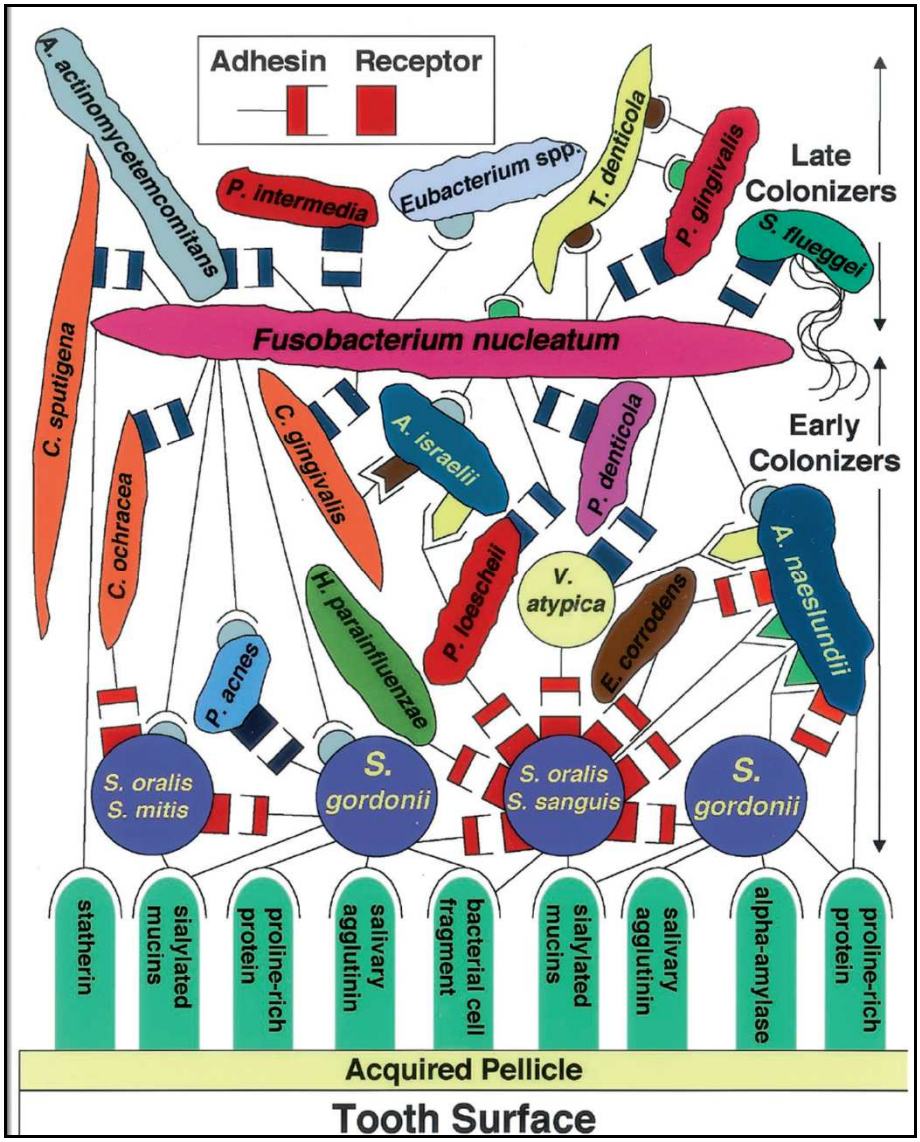

Figure 8. Spatiotemporal model of oral bacterial colonization of the tooth surface.

This model shows the recognition of salivary pellicle receptors by early colonizing bacteria and coaggregations between early colonizers, fusobacteria, and late colonizers of the tooth surface. Each coaggregation depicted is known to occur in a pairwise test. Collectively, these interactions are proposed to represent development of dental plaque according to Kolenbrander et al. (Kolenbrander et al. 2002). Starting at the bottom, primary colonizers bind via adhesins (round-tipped black line symbols) to complementary salivary receptors (blue-green vertical round-topped columns) in the acquired pellicle coating the tooth surface. Secondary colonizers bind to previously bound bacteria. Sequential binding results in the appearance of nascent surfaces that bridge with the next coaggregating partner cell. Several kinds of coaggregations are shown as complementary sets of symbols of different shapes. One set is depicted in the box at the top. Proposed adhesins (symbols with a stem) represent cell surface components that are heat inactivated (cell suspension heated to 85°C for 30 min) and protease sensitive; their complementary receptors (symbols without a stem) are unaffected by heat or protease. Identical symbols represent components that are functionally similar but may not be structurally identical. Rectangular symbols represent lactose-inhibitable coaggregations. Other symbols represent components that have no known inhibitor. The bacterial strains shown are *Actinobacillus actinomycetemcomitans, Actinomyces israelii, Actinomyces naeslundii, Capnocytophaga gingivalis, Capnocytophaga ochracea, Capnocytophaga sputigena, Eikenella corrodens, Eubacterium spp., Fusobacterium nucleatum, Haemophilus parainfluenzae, Porphyromonas gingivalis, Prevotella denticola, Prevotella intermedia, Prevotella loescheii, Propionibacterium acnes, Selenomonas flueggei, Streptococcus gordonii, Streptococcus mitis, Streptococcus oralis, Streptococcus sanguis, Treponema spp.*, and *Veillonella atypical* (Kolenbrander et al. 2002).

1.3.2 Development of caries

The tooth enamel is build up of hydroxylapatite [$Ca_{10}(PO_4)_6(OH)_2$] and its stability is dependent on pH and the free active concentrations of calcium and phosphate ions. When the pH is lowered beneath the critical pH of ~5.5 the enamel starts to demineralise, while above this pH it tends to remineralise. Frequent demineralisation causes cavities to form in the teeth, called tooth decay or caries lesion. The neutral pH and the buffer capacity of saliva shift the equilibrium towards remineralisation (Figure 9). Conditions of low pH can be achieved e.g. by impaired saliva flow or by the fermentation of dietary sugars by acidogenic bacteria (like mutans streptococci) which results in the formation of acidic end-products.

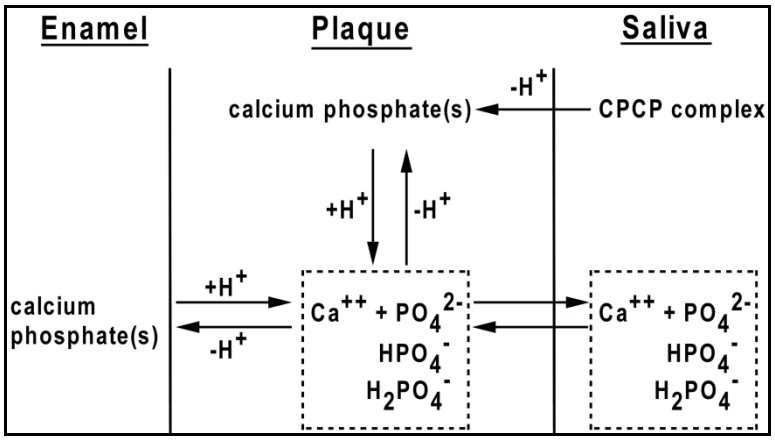

Figure 9. Equilibrium of de- and remineralisation of the tooth enamel.
Role of saliva in the various calcium phosphate reactions that occur at the tooth-plaque-saliva interface. Saliva provides calcium and phosphate (i) as ions and (ii) as part of a calcium phosphate carbohydrate protein complex (i.e., salivary precipitin). Both help to provide a continuous supply of calcium and phosphate for plaque and tooth mineralization. At acidic pH, movement of calcium and phosphate from tooth and plaque to saliva is favoured, whereas at alkaline pH, the reverse is favoured. The calcium and phosphate ions produced in the plaque from plaque calcium phosphate during acid formation are in a position to suppress or retard tooth solubilization by mass action as well as to facilitate tooth remineralisation (Kleinberg 2002).

Paradigm shift in the concept of caries development

The current view of caries progression has evolved through two paradigm shifts (Fejerskov 2004). The "*specific plaque hypothesis*" proposed that out of the diverse collection of bacteria in dental plaque, only a very limited number of species are involved in caries outcome, including *S. mutans* (Loesche 1976). This hypothesis was based on the argument that mutans streptococci (MS) could not be found in some patients without caries, using cultivation methods, and thus it was suggested that one has to be infected by MS to get caries (Fejerskov 2004). However, although MS are implicated with caries, caries lesions can also develop in

the absence of MS. Moreover, MS can persist on tooth surfaces without evidence of caries progression (Marsh et al. 1989). This lead to an alternative view of the development of periodontal diseases which was presented in the "*non-specific plaque hypothesis*". It proposes that different combinations of common plaque bacteria, rather than just a single species, and their interactions produce the conditions that are necessary to cause dental diseases, since using cultivation based methods bacteria isolated from caries lesions were also found on healthy sites (Theilade 1986). What has not been considered in that hypothesis is that the predominant species recovered from diseased sites are different from those found in health, leading to the development of the "*ecological plaque hypothesis*" (Marsh 2003).The use of more sensitive detection methods (PCR, *in-situ* hybridization, ELISA) demonstrated the occurrence of several pathogens associated with dental diseases at a wide range of sites, suggesting that plaque mediated diseases, such as caries, are due to an imbalance of the resident microflora resulting in an enrichment of pathogenic bacteria within the microbial community. This enrichment is due to selective pressures that result from disturbances in the homeostatic mechanisms (Marsh 2003). The predominant species in mature smooth surface plaque belong to *Actinomyces* and *Streptococcus*, most of which are non-MS (Socransky et al. 1998) (Figure 10). Both species are able to liberate sugars from the salivary glycoproteins and contain specific receptor proteins to adhere to the acquired pellicle, giving them a selective advantage compared to MS and other plaque bacteria (Takahashi and Nyvad 2008). Due to frequent intake of sugars, acidification of the biofilm occurs due to the acidogenicity of non-MS. Non-MS are heterogeneous with respect to their aciduric properties and thus the microbial homeostasis will shift towards "low-pH" non-MS. A similar mechanism is suggested for *Actinomyces* (Figure 10) (Takahashi and Nyvad 2008). However, with ongoing acidification due to sugar intake, MS and other aciduric (cariogenic) bacteria are more competitive since they are more resistant to acid than "low-pH" non-MS and their proportion will increase (Figure 10). It is important to note that cultivation based methods are the basis for all these observations and that the role of the presently uncultivated bacteria for these processes is currently unknown. The increased growth of strong acidogenic and aciduric bacteria leads to more and more phases during which the pH is below the critical pH, thus favouring again the growth of cariogenic bacteria and subsequent demineralisation of the enamel (Marsh 2003, Marsh 2006, Takahashi and Nyvad 2010).

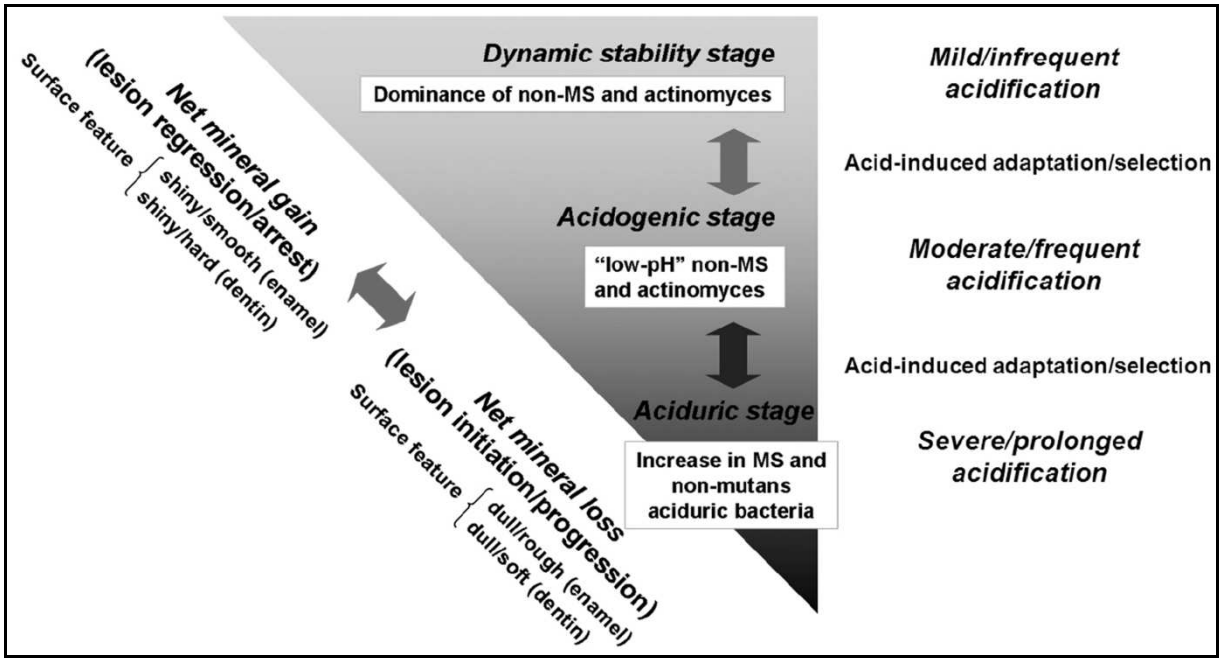

Figure 10. The caries process according to an extended ecological plaque hypothesis.
Under conditions of a normal (low sugar) diet, non-mutans streptococci (MS) and actinomyces are the key players to maintain pH stability. Aciduric bacteria are in the minority and clinical not relevant. This ratio shifts towards the presence of aciduric bacteria with frequented sugar intake and acid-induced adaptation processes of aciduric bacteria. This shift is concomitant with prolonged phases of acidification and a net mineral loss (demineralisation) of the tooth enamel and the initiation of caries lesions (Takahashi and Nyvad 2010).

1.3.3 Virulence determinants of *S. mutans*

Cariogenic bacteria, such as *S. mutans*, have a key role in the formation of dental plaque and in the demineralisation of the tooth enamel and thus in the initiation and progression of dental caries. *S. mutans* possesses certain characteristics that are not only important in the process of caries progression but also to cope with other bacteria for its ecological niche (Banas 2004, Kuramitsu 1993, Li et al. 2002):

(I) the ability to utilize a broad spectrum of dietary sugars to produce extracellular polysaccharides (EPS) that contribute to the plaque biofilm matrix and promote bacterial colonization,
(II) possesses high and low affinity sugar transport systems operating over a wide range of conditions, ensuring that sugar uptake can be carried out even at low pH and at low sugar concentrations,
(III) the ability to produce and store intracellular polysaccharides (IPS) when carbohydrates are in excess. This ensures metabolism and thus acidification even in the absence of dietary carbohydrates,

(IV) a glycolytic pathway that efficiently operates at low pH values and ensures growth under strong acidified conditions,
(V) the ability to grow and to survive in a low pH environment (pH ~ 4) (aciduricity) due to the induction of its acid tolerance response (ATR),
(VI) produces various bacteriocins (named mutacins) that exhibit anti-microbial activity against it surrounding competitors, and
(VII) possesses natural competence, allowing the acquisition of fitness enhancing DNA.

Remarkable of *S. mutans* are its acidogenic and aciduric properties. Under conditions of sugar increased diet, these virulence traits help to outcompete non acid-tolerating bacteria and will increase the number of MS significantly from <2% of the initial streptococcal population under conditions of normal diet up to 30% of the total microflora in cavitated lesions determined with cultivation based methods (Takahashi and Nyvad 2010).

The acid tolerance response (ATR) of *S. mutans*

The acid tolerance response of *S. mutans* strongly contributes to its ability to cope with low pH conditions that are produced during fermentation of dietary sugars by acidogenic bacteria. The ATR is induced optimally at above lethal pH (pH 5-5.5) and serves to protect the cells when the pH is lowered further to lethal pH conditions (pH < 3-4). The ATR includes the following traits (Lemos and Burne 2008, Belli and Marquis 1991, Len et al. 2004a, Len et al. 2004b, Svensater et al. 1997):

(a) increased F0F1 ATPase activity to translocate protons across the cell membrane,
(b) shift to a lower pH optimum for enzymes of sugar transport and glycolysis,
(c) increased glycolytic activity concomitant with a shift to a homo-fermentative metabolism, (producing mainly lactacte as end-product), having a higher yield of ATP than hetero-fermentative metabolism (producing lactate, acetate, ethanol).
(d) synthesis of general stress-responsive genes (i.e. chaperones, proteases, DNA repair enzymes),
(e) reduction of the membrane proton permeability through the synthesis of monounsaturated fatty acids and ,
(f) use of more specific reactions that contribute to a de-acidification of the cytoplasm like the agmatine deiminase system or malolactic fermentation.

The final pH values in cultures of non-mutans streptococci when grown with sugars are diverse, ranging from pH 4-5.2, whereas those of mutans streptococci stabilize at an average level of around pH 4 (Takahashi and Nyvad 2008). Since the ATR of *S. mutans* is stronger

than in other bacteria and protects more against the low pH values that are obtained from the overall metabolic activity of acidogenic bacteria, acid stress helps *S. mutans* to outcompete less aciduric bacteria under prolonged conditions of low pH.

Biofilm formation by *S. mutans*

The formation of an extracellular polysaccharide matrix (EPS) is the primary basis for surface adhesion and biofilm formation of *S. mutans*. The EPS is comprised mainly of glucan and fructan polymers that are formed due to the action of glycosyltransferases (GTF) and fructosyltransferases (FTF) using sucrose as substrate. Glucan is believed to be the major factor that promotes adherence of *S. mutans* to tooth surfaces and aggregation of the bacterial cells within a biofilm (Munro et al. 1995). *S. mutans* possesses three GTF enzymes. Both GTF-I (encoded by *gtfB*) and GTF-SI (encoded by *gtfC*) are cell wall associated and produce water-insoluble glucans (called mutan). However, water-soluble glucan (called dextran) to a small extent is produced by GTF-SI, too. The extracellular enzyme GTF-S (encoded by *gtfD*) synthesizes predominantly dextran. It has been shown that in the presence of sucrose the loss of GTF-I most strongly affected adherence to a glass support (Tamesada et al. 2004).
In spite of their individual functions the highest levels of sucrose dependent adhesion were found in a cooperative action of all three enzymes at a optimal ratio of 5 GTFI : 0.25 GTF-SI : 1 GTF-S (Ooshima et al. 2001). Further necessary to achieve the complete architecture of the biofilm are the glucan binding proteins (Gbp), of which four have been characterized in *S. mutans*. Individual deletion of each glucan binding protein altered the biofilm structure in its own way but a significant reduction in biofilm depth was concomitant with every single deletion. The most dominant effect was displayed by GbpC since it is cell wall associated and thus mediates a bond between bacteria and glucan. The released glucan binding proteins GbpA and GbpB contributed more to the scaffolding allowing *S. mutans* to build a multilayered biofilm distant from the substratum (Lynch et al. 2007). Interestingly, glucan binding proteins have been identified only among mutans streptococci (Banas and Vickerman 2003), emphasizing their strong contribution to the development of the biofilm matrix of mutans streptococci.

1.3.4 Quorum sensing in dental plaque and by *S. mutans*

The dental plaque biofilm comprises hundreds of different species that live together in close proximity, thus it is likely that diverse types of interactions between different bacterial species and within the same species exist. Here, I will concentrate on signalling mechanisms.

The role of autoinducer-2 in dental plaque

Autoinducer-2 has attracted a great deal of attention since it was shown to mediate interspecies communication in members of the dental plaque and is found to be widespread among Gram-negative and Gram-positive bacteria (Sun et al. 2004). Rickard *et al*. demonstrated that both *Actinomyces naeslundii* T14V and *Streptococcus oralis* 34 exhibited a weak mono-species biofilm when grown alone in saliva-fed flowcells. In contrast, co-inoculation resulted in a thick biofilm, which was severely disturbed when the *luxS* gene of *S. oralis* was deleted. Complementation with chemically synthesized AI-2 in a narrow concentration range restored the dual-species biofilm, demonstrating the mutualistic partnership of both strains in the form of AI-2 signalling (Rickard et al. 2006). In another oral commensal, *Aggregatibacter actinomycetemcomitans,* AI-2 was shown to be essential for the formation of mono-species biofilms (Shao et al. 2007).
S. mutans was shown to produce and to respond to AI-2 (Thiel et al. 2009, Sztajer et al. 2008). In the study of Sztajer *et al*. it was shown that addition of AI-2 to a *luxS* mutant changed the expression of 59 genes. Among the affected genes, transcriptional regulators such as MleR were present suggesting a regulatory role for AI-2 in *S. mutans*, which has to be elucidated in more detail.

The role of competence stimulating peptides (CSP) in dental plaque

The CSP pheromone has been originally identified in *S. pneumoniae* and was shown to be involved in the regulation of competence (Havarstein et al. 1995). CSP molecules of streptococci are small unmodified peptides, ranging from 14 to 25 residues in length. The structure of *S. mutans* CSP has been elucidated and revealed that the last 5 amino acids of the C-terminus were crucial for activation of the signal transduction pathway and that this motif is widespread in the CSPs of other streptococci, indicating a similar function. The remaining peptide was required for receptor binding and is not conserved (Syvitski et al. 2007). The presence of such peptides was shown to be abundant in oral streptococci (Martin et al. 2006) and they were shown to be involved in the regulation of competence and bacteriocin production in various oral streptococci (Kreth et al. 2005, Vickerman et al. 2007a).

The bacteriocins of *S. mutans* (termed mutacins) are generally divided into two groups: (i) the posttranslationally modified, lantihionine-containing (lantibiotic) mutacins and (ii) the unmodified peptide mutacins. Different isolates of *S. mutans* possess different sets of mutacins (Kamiya et al. 2008). The genome of *S. mutans* UA159, which was used in our study, encodes only for nonlantibiotic mutacins. These mutacins are produced as a peptide containing a leader peptide with a highly conserved double-glycine (GG) motif. Proteolytic

cleaving at this site and thus maturation of the peptide occurs during export using an ATP-binding cassette (ABC) transport system. Bacteriocins are different from peptide pheromones/autoinducers. Their sizes ranges between 37-45 residues and they consist of a highly conserved hydrophilic and charged N-terminus harbouring a consensus sequence. The C-terminal part is more hydrophobic and/or amphiphilic and more variable (Drider et al. 2006). The targets for nonlantibiotic mutacins are not well characterized but the involvement of the mannose specific phosphotransferase system has been suggested (Nes et al. 2007).

CSP molecules are very divergent and are not conserved in different species of streptococci. So far, it is not known whether one species of *Streptococcus* responds to the CSP of another one. Previously, Jarosz *et al*. reported that the CSP of *S. mutans* inhibited hyphae formation in *Candida albicans* at physiological concentrations, thus it cannot be excluded that further work will demonstrate bacterial interspecies communication by CSPs (Jarosz et al. 2009). Intra-species signalling using CSP results often in the production of bacteriocins as it is the case for *S. mutans*. Since bacteriocins are mainly active against close relatives it would be advantageous for bacteria living in close proximity to the producer strain to interfere with CSP mediated induction of bacteriocin production. It was reported that *S. gordonii, S. sanguinis, S. mitis, S. oralis* and *S. salivarius* were able to inhibit mutacin production by degrading *S. mutans* CSP, demonstrating the interaction and competition between oral streptococci in dental plaque (Tamura et al. 2009, Wang and Kuramitsu 2005).

The specific role of CSP in *S. mutans*

The CSP pheromone is a very important signalling molecule of *S. mutans* since it controls bacteriocin production, competence development, biofilm formation and has been shown to modulate the aciduricity (Kreth et al. 2006, Li et al. 2001a, Li et al. 2002).
The *comC* gene encodes for the pre-cursor of the competence stimulating peptide (CSP), which is cleaved and exported through the ComAB ABC transporter into the environment. After exceeding a certain threshold the mature CSP binds to its receptor, the histidine kinase (HK) ComD which forms a two component system with its cognate response regulator (RR) ComE. Activation of ComD leads to an autophosphorylation on a conserved histidine residue followed by transfer of the phoshporyl group to an aspartate residue within the receiver domain of the response regulator ComE (Laub and Goulian 2007, Li et al. 2002) (Figure 11). This leads to conformational changes of the output domain of the response regulator, resulting in altered DNA binding capacities and thus different transcriptional activity (Laub and Goulian 2007, Li et al. 2002). CSP activated ComE activates the expression of different bacteriocin related genes (the so-called early genes), including the potent mutacin IV, mutacin

V and its cognate immunity protein *cipI*, due to the presence of a direct repeat motif (ComE box) in their promoter regions (Kreth et al. 2006, van, Jr. 2005).
In contrast to *S. pneumoniae*, no ComE box is present in the promoter region of *comC*, *comDE* or *comX*, suggesting a different activation of the alternate sigma factor, ComX (SigX). Another difference compared to *S. pneumoniae* is that the highest expression of *comE* and *comX* is achieved 2 hours after induction by CSP and that only a subpopulation starts the expression of SigX (Perry et al. 2009b). Currently the mechanism that triggers the bifurcation of the culture into different subpopulations is not known. Recently, a transcriptional regulator, ComR, was identified which was shown to be required for the direct activation of SigX (Mashburn-Warren et al. 2010). The binding motif of ComR, a inverted repeat motif, was found in the promoter region of ComX and ComS. ComS was identified to be a posttranslationally modified peptide (termed XIP), which is needed for the transcriptional activity of ComR and is encoded in the intergenic region adjacent to ComR. It is currently suggested that all signals that modulate competence in *S. mutans* are transmitted to ComR but it is not known how this is achieved (Figure 11) (Mashburn-Warren et al. 2010).

Activation of ComR leads to an auto-catalytic production of ComS. ComS is secreted through a currently unknown transporter, posttranslationally modified in the outer environment – resulting in the mature XIP peptide, and transported back into the cell via the Opp oligopeptide permease. ComR in conjunction with XIP activate the expression of the alternate sigma factor SigX, which directly induces the expression of the late competence genes, including genes required for DNA uptake and processing (Mashburn-Warren et al. 2010, Johnsborg et al. 2007). The CSP signalling circuit is one of four components that have been shown to influence the expression of SigX and are illustrated in a model in figure 11. Moreover, it was shown that a low pH environment or sub-inhibitory concentrations of some antibiotics induce the expression of *comC* and subsequent activation of competence (Perry et al. 2009b). The HdrRM system was shown to induce competence under conditions of biofilm growth but the discovery of a signalling molecule remains elusive (Okinaga et al. 2010b, Okinaga et al. 2010a). However, the presence of the CSP pheromone above its threshold triggers the activation of mutacins and competence. Perry *et al*. showed that mutacin V is a self-acting bacteriocin that accumulated intracellularly and triggers autolysis of some cells. Of particular interest was their observation that only cells that expressed SigX were susceptible for the fluorescence dye propidium iodide, which stains membrane damaged cells, and thus they suggested that the CSP dependent pathway does not contribute to the uptake of fitness-enhancing DNA, as CSP induced cells are killed due to the mutacin V mediated autolysis (Perry et al. 2009b). They demonstrated that autolysis results in the release of DNA, which was used to establish *S. mutans* biofilm architecture (Perry et al. 2009a). They further suggested that the uptake of DNA is accomplished by cells that become competent through a CSP independent pathway, which does not activate mutacin production and thus autolysis

(Perry et al. 2009b). Many questions still have to be addressed to clarify the competence development in *S. mutans*.

Addition of CSP triggers competence development in *S. mutans* and this state lasts for about 3-4 hours, according to its ability to take up DNA from the environment (Petersen and Scheie 2010). Currently, no information is present about any mechanism that mediates the exit from the competence state. The genome of *S .mutans* encodes two Spx proteins like *S. pneumoniae*. One of these paralogues (SpxA) has been shown to be essential to cope with various stresses, whereas the second protein (SpxB) showed only a small contribution towards stress tolerance. Interestingly, SpxB is located adjacent to two import late competence genes and thus it might fulfil a similar function as SpxA1 in *S. pneumoniae*, and might be involved in leaving the competent state.

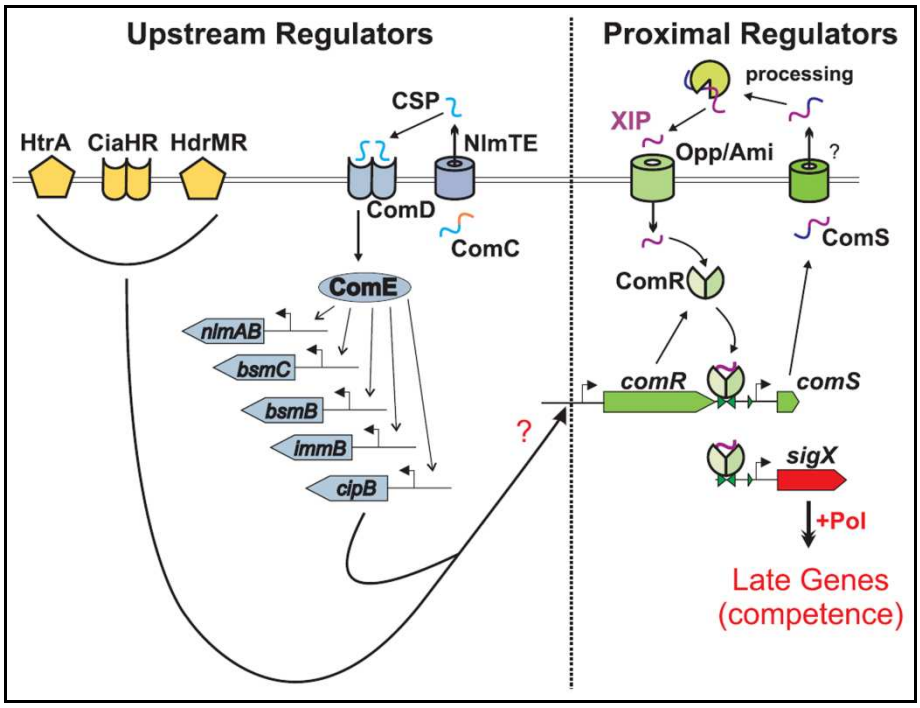

Figure 11. Current model of competence development in *S. mutans*

The action of several upstream regulators is integrated to the transcriptional regulator ComR, which activates in conjunction with the mature peptide of ComS the expression of ComS and SigX (alternate sigma factor ComX). SigX directs the RNA polymerase to the late competence gene promoters and activates their expression. The CSP peptide is secreted and processed by the ComAB transporter (NlmTE) and activates the two component system ComDE. The response regulator ComE activates bacteriocin related genes, including mutacin V, which establish an undefined link to *sigX* expression. The components in secretion and processing of ComS are unknown. The matured peptide XIP (sigX inducible peptide) is internalised through the Opp oligopeptide permease transporter (Mashburn-Warren et al. 2010).

1.4 Aim of the work

The aim of this study was to identify and to characterize new genes/proteins or mechanisms that are involved in the quorum sensing regulation of *Streptococcus mutans*. Of particular interest was to elucidate the unknown role of the autoinducer-2 signalling molecule and to obtain more detailed insights into the well characterized competence stimulating peptide (CSP) mediated activation of competence and bacteriocin production.

1. **Characterization of MleR.**
   The study of Sztajer *el al.* showed that expression of the transcriptional regulator *mleR* was induced upon addition of AI-2 (Sztajer et al. 2008). To confirm this observation, the expression of *mleR* was analysed in more detail but no role for AI-2 could be shown. Since it was found that MleR is involved in the regulation of malolactic fermentation, its contribution to the aciduricity of *S. mutans* was investigated. Moreover, the expression of *mleR* and its target gene, *mleS*, was analysed under various conditions. Binding studies of MleR and DNA were carried out to confirm its role as transcriptional activator.

2. **Genetic, physiological and chemical complementation of a *luxS* mutant of *S. mutans***
   Deletion of *luxS* leads to a disruption of the AMC and to a lack of AI-2. To complement the metabolic defects of a disrupted AMC the alternative enzyme SAH-hydrolase was introduced into a *luxS* mutant of *S. mutans*. Complementation with pure chemically AI-2 was carried out to elucidate the influence of AI-2 on global gene expression (microarray analysis) and important virulence traits of *S. mutans*, such as biofilm formation, competence and mutacin production. Since it turned out that the *luxS* mutant contained secondary mutations a new mutant and a plasmid for genetic complementation was constructed. The influence of both *luxS* deletion and genetic complementation, on global gene expression and *S. mutans* virulence traits, have been analysed.

3. **Single cell analysis of CSP mediated competence development in *S. mutans*.**
   CSP dependent competence development in *S. mutans* is a bistable process and is restricted to a subpopulation. To reveal differences between competent and non-competent cells a microarray analysis of both subpopulations was carried out. Cells were separated using flow cytometry guided by a GFP reporter for the alternative

sigma factor, SigX. To confirm on phenotypic level that competent cells are transformable, DNA uptake was visualized for individual cells. For confirmation of the microarray results two additional GFP reporter strains for ComE and mutacin V were constructed and analysed using fluorescence microscopy and flow cytometry. To confirm the correlation of cells becoming competent by addition of CSP and autolysis as reported by Perry *et al*. (Perry et al. 2009b), cells expressing GFP were counterstained with propidium iodide and analysed on single cell level.

4. **Characterization of SpxB and its role in competence development**
   Activation of competence by CSP is a transient process but no mechanism for its de-activation are known. Since Spx proteins have been shown to control the exit from competence in *S. pneumoniae* and *B. subtilis* (Nakano et al. 2001, Turlan et al. 2009), we investigated the influence of the SpxB protein in the competence development in *S. mutans*. To obtain insights in its regulation, mutants defective in several histidine kinases and regulators have been investigated for their *spxB* expression.

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CHAPTER II

Characterization of *mleR*, a positive regulator of malolactic fermentation and part of the acid tolerance response in *Streptococcus mutans*

André Lemme, Helena Sztajer, Irene Wagner-Döbler

Helmholtz-Centre for Infection Research, Research Group Microbial Communication, Inhoffenstr. 7, D-38124 Braunschweig.

2 Chapter II - Characterization of *mleR*, a positive regulator of malolactic fermentation and part of the acid tolerance response in *Streptococcus mutans*

2.1 Abstract

One of the key virulence determinants of *Streptococcus mutans*, the primary etiological agent of human dental caries, is its strong acid tolerance. The acid tolerance response (ATR) of *S. mutans* comprises several mechanisms that are induced at low pH and allow the cells to quickly adapt to a lethal pH environment. Malolactic fermentation (MLF) converts L-malate to L-lactate and carbon dioxide and furthermore regenerates ATP, which is used to translocate protons across the membrane. Thus, MLF may contribute to the aciduricity of *S. mutans* but has not been associated with the ATR so far.
Here we show that the malolactic fermentation (*mle*) genes are under the control of acid inducible promoters which are induced within the first 30 minutes upon acid shock in the absence of malate. Thus, MLF is part of the early acid tolerance response of *S. mutans*. However, acidic conditions, the presence of the regulator MleR and L-malate were required to achieve maximal expression of all genes, including *mleR* itself. Deletion of *mleR* resulted in a decreased capacity to carry out MLF and impaired survival at lethal pH in the presence of L-malate. Gel retardation assays indicated the presence of multiple binding sites for MleR. Differences in the retardation patterns occurred in the presence of L-malate, thus demonstrating its role as co-inducer for transcriptional regulation.
This study shows that the MLF gene cluster is part of the early acid tolerance response in *S. mutans* and is induced by both low pH and L-malate.

2.2 Introduction

S. mutans is considered the major etiological agent of dental caries due to its strong aciduric and acidogenic capacities. During the metabolism of dietary carbohydrates and subsequent formation of acid end-products, acidogenic bacteria can shift the plaque pH to 4 or lower within minutes and can retain it at this value for up to one hour, depending on the age of the plaque biofilm (Igarashi et al. 1981, Jensen et al. 1982, Jensen and Wefel 1989, Schachtele and Jensen 1982). Demineralisation of the tooth enamel caused by low pH is the beginning of caries development. To withstand these pH fluctuations and to compete with other oral

bacteria *S. mutans* has evolved an effective acid tolerance response (ATR). The ATR is induced under acidic conditions and has an optimal pH between 5.5-5. Several proteomic studies showed that more than sixty proteins were involved in this response and that many of them appeared within the first 30 minutes after acid shock, whereas full induction occurred after 90-120 minutes (Hamilton and Svensater 1998, Len et al. 2004a, Dashper and Reynolds 1992, Svensater et al. 1997).

General determinants are the induction of general stress proteins, the reduction of membrane proton permeability, increased glycolytic activity and a shift to homo-fermentative metabolism, resulting in elevated lactate production. Anabolic reactions are in return down-regulated, which results in slower growth and lower cell yield (Belli and Marquis 1991, Len et al. 2004a, Len et al. 2004b, Svensater et al. 1997). The concomitant surplus of ATP is used to drive the H^+/ATPase, which leads to an increased translocation of protons across the membrane. More specific reactions that contribute to the aciduricity are e.g. the agmatine deiminase system (AgDS). Agmatine is secreted by other bacteria in response to low pH but is internalised and deaminated by *S. mutans* to ammonia and carbamoylputrescine. The latter is further decarboxylated to putrescine, yielding carbon dioxide and ATP, which again can be used for proton extrusion (Griswold et al. 2004).

Another mechanism for gaining ATP is malolactic fermentation (MLF), which is a secondary fermentation that lactic acid bacteria can carry out when L-malate is present in the medium. Its biochemical properties have been studied in detail because of the considerable biotechnological interest, since it occurs after the alcohol fermentation during wine making affecting the flavour of the wine. In MLF the dicarboxylic acid L-malate is converted to L-lactate and carbon dioxide by the malolactic enzyme (MLE) in a two step reaction without releasing intermediates. Since malic acid (pKa = 3.4, 5.13) is a stronger acid than lactic acid (pKa = 3.85) decarboxylation of L-malate leads to an alkalinization of the cytoplasm. This effect is further enlarged by diffusion of H_2CO_2/CO_2 out of the cell into the gas phase. The concomitant pH gradient drives the electrogenic malate/lactate antiporter and is coupled to ATP synthesis, which is used to maintain the intracellular pH more alkaline than the environment by extrusion of protons (Lemos and Burne 2008, Poolman et al. 1991). *S. mutans* UA159 possesses a malolactic fermentation gene cluster, that is oriented in opposite direction to the putative regulator *mleR* (Ajdic et al. 2002). A homologue of this regulator was the first lysR-type transcriptional regulator (LTTR) described in Gram positive bacteria and was shown to positively regulate MLF in *Lactococcus lactis*. A seven-fold induction of L-malate decarboxylation activity and a three-fold increase of gene expression determined by a *mleR-lacZ* fusion was observed in the presence of L-malate (Renault et al. 1989). However, in *Oenococcus oeni* malolactic fermentation activity was not enhanced by the presence of MleR or L-malate (Labarre et al. 1996). Recently Sheng and Marquis showed that *S. mutan*s possesses MLF activity with a pH optimum of pH 4 in planktonic cells (Sheng and Marquis 2007). Significant intracellular ATP maintenance and enhanced protection against lethal pH

values were observed in the presence of L-malate (Sheng and Marquis 2007). Since this study showed that MLF has a great impact on the aciduric capacities of *S. mutans*, we were interested if this mechanism is part of the general ATR of the cell or if it is specifically induced by MleR and the presence of L-malate. Deletion of *mleR* and luciferase reporter strains for *mleR* and *mleS* and RT-PCR revealed insights into the expression and regulation of the *mle* gene cluster and especially the effect of pH. Electrophoretic mobility shift assays (EMSA) indicated several binding sites for the MleR protein which were influenced by the presence of L-malate. Moreover we investigated the role of MleR for the ability of *S. mutans* to withstand acid stress.

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2.3 **Results**

2.3.1 Analysis of the *mle* locus by RT-PCR and EMSA

In the genome of *S. mutans* UA159 (Ajdic et al. 2002), the lysR type transcriptional regulator MleR is orientated opposite to a gene cluster encoding the malolactic enzyme (*mleS*), a malate permease (*mleP*), and a oxalate decarboxylase (*oxdC*), respectively. Additionally a putative prophage repressor is inserted between *mleR* and *mleS* (Figure 12). This insertion is unique for the oral *streptococci* (*S. mutans* UA159, *S. gordonii* str. Challis CH1 and *S. sanguinis* SK36) among all sequenced *Lactobacillales*. Adjacent to the genes involved in malolactic fermentation is the gene *oxdC* encoding the oxalate decarboxylase which catalyses the conversion of oxalate to formate and CO_2. This gene is unique for *S. mutans* UA159 among all sequenced *Lactobacillales*. RT-PCR disclosed that it is co-transcribed with *mleS* and *mleP* since it was possible to amplify overlapping fragments of all three genes (Figure 12A). The putative gluthatione reductase (SMU.140) located downstream of *oxdC*, which is involved in the removal of reactive oxygen species, could not be assigned to the same operon by the use of RT-PCR. Applying the Electrophoretic Mobility Shift Assay (EMSA) (Figure 12B) using a DNA fragment covering almost the complete intergenic sequence (IGS) between *mleR* and SMU.136c (EMSA 1) resulted in one retarded complex, indicating one binding site for MleR in this intergenic region. Elongation of the DNA fragment (EMSA 2) to include the 3' end of SMU.136c, produced two retarded bands, suggesting an additional binding site at the 3' end of SMU.136c. The presence of 5 mM L-malate in both EMSA reactions gave the same banding pattern. However, the extent of the shift was slightly reduced.
Using the complete coding sequence of SMU.136c (EMSA 3) resulted in one retarded complex, confirming the presence of one binding site for MleR in this gene.

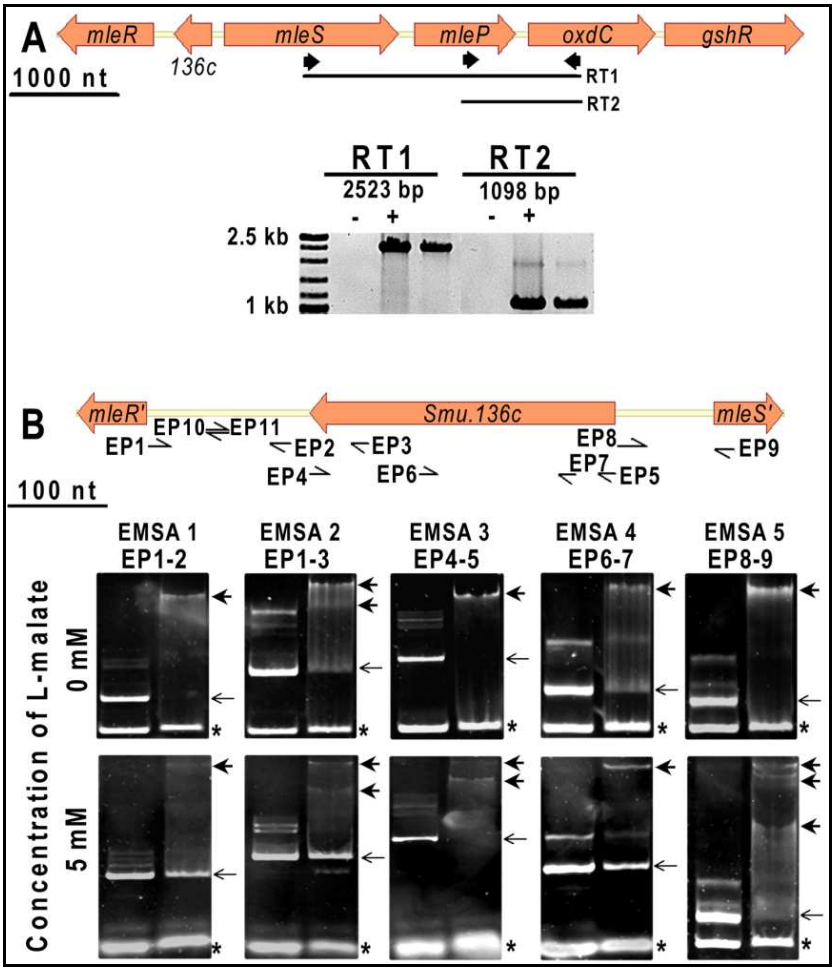

Figure 12. Genetic organization of the mle locus.

A: RT-PCR analysis of mRNA transcripts. The solid arrows indicate the primers used for RT-PCR. The minus RT control is assigned with ”–“; the positive control, using genomic DNA, is assigned with “+”. **B**: Gelshift analysis of the region between mleS and mleR. Arrows indicate primers that were used to amplify PCR products, that were subsequently used for EMSA. Primers are designated at their 5’ end. The box shows a representative selection of gel shift assays with the respective fragment in the presence or absence of L-malate. Thin arrows indicate DNA fragments in the absence of protein. Bold arrows indicate DNA in complex with MleR. Competitor DNA is marked with an asterix. For all EMSAs, 1x binding buffer was loaded on the left and MleR protein on the right lane. In all EMSAs without malate, an internal fragment of mleS was used as competitor DNA. In EMSAs with malate the fragment within the IGS of mleR and SMU.136c, generated by hybridising primers EP10/11 was used (except for EMSA 5, where the internal fragment of mleS was added). Addition of L-malate to the binding reaction changed the pattern in this case and produced two retarded fragments. Truncation of the 3’ end of SMU.136c (EMSA 4) resulted only in one retarded fragment, independent of L-malate. The data show the presence of at least two binding sites for MleR within SMU.136c. One site is located within fragment EP 6-7 (EMSA 4) presumably binding the apo form of MleR and another one is located at the 3`end of SMU.136c and appears to need the co-inducer bound form of MleR. The intergenic sequence upstream of mleS (EMSA 5) produced one retarded complex in the absence and three complexes in the presence of 5 mM L-malate. Thus, within this IGS also several binding sites for different forms of MleR exist. Using internal DNA fragments of mleS or mleR (data for mleR not shown) or a sequence within the IGS of mleR and SMU.136c (primers 137qF/R) did not produce complexes with the MleR protein under the tested condition, thus confirming the specificity of the DNA-protein interaction. Incubation of all used DNA fragments with BSA instead of MleR resulted in no retardation (data not shown).

2.3.2 Involvement of *mleR* in MLF activity

It was previously shown that *S. mutans* UA159 was able to carry out malolactic fermentation (Sheng and Marquis 2007). To determine if the putative regulator MleR is involved in the regulation of the MLF gene cluster a knockout mutant of *mleR* was constructed, by replacing an internal part (amino acids 27-275) of the gene with an erythromycin resistance cassette, amplified from another strain (Sztajer et al. 2008). *S. mutans* wildtype cells showed highest MLF enzyme activity in the presence of 25 mM L-malate at the beginning of the stationary phase (Sheng and Marquis 2007). Under these conditions, we observed a significant reduction of MLF activity of the Δ*mleR* mutant compared to the parental strain, indicating a positive regulation of the *mle* genes by MleR (Table 2).
After one hour the wild type strain converted or internalised over 40% of the added L-malate. For the mutant lacking the MleR regulator only a 1% reduction of the added malate within one hour was observed. Furthermore, internalisation and decarboxylation of the stronger malic acid to lactic acid leads to a considerable increase of the external pH (Table 2).

Table 2. Malolactic fermentation activity for the wildtype and the Δ*mleR* mutant.

	L-malate concentration [mg/ml]		pH-value	
Time	WT	Δ*mleR*	WT	Δ*mleR*
0 min	5.53	5.63	6.4	6.34
20 min	4.87	5.61	6.7	6.32
40 min	2.77	5.59	6.9	6.43
60 min	2.34	5.42	7.2	6.52
12 hours	1.26	3.51	8.2	7.32

The capability to carry out malolactic fermentation was determined by measuring the L-malate concentration and the pH of the supernatant of cultures grown to late exponential phase (OD ~1.3). The values represent the average of two independent experiments. The standard deviation was less than 5%.

However, after 12 hours of incubation a reduction of 78% and 38% of the added L-malate was observed in the wildtype and the Δ*mleR* mutant, respectively, indicating a basal level of MLF enzyme activity in the absence of MleR.

2.3.3 Transcription of *mle* genes during growth

To obtain better insights into the transcriptional regulation of the MLF gene cluster and *mleR* itself, firefly luciferase reporter plasmids were constructed. The upstream sequences of *mleR* and *mleS* containing the putative promoter sequences were cloned in front of a promoterless luciferase gene and then integrated into the genome of the wildtype and the Δ*mleR* mutant by single homologous recombination, respectively. Luciferase activity was monitored during growth in the absence of L-malic acid (Figure 13). The highest activity for both promoters was observed at the transition to the stationary phase, with an external pH between 5.8 and 6.1. This was true for the parental strain and the Δ*mleR* mutant, indicating that both transcriptional units might be controlled by acid inducible promoters.

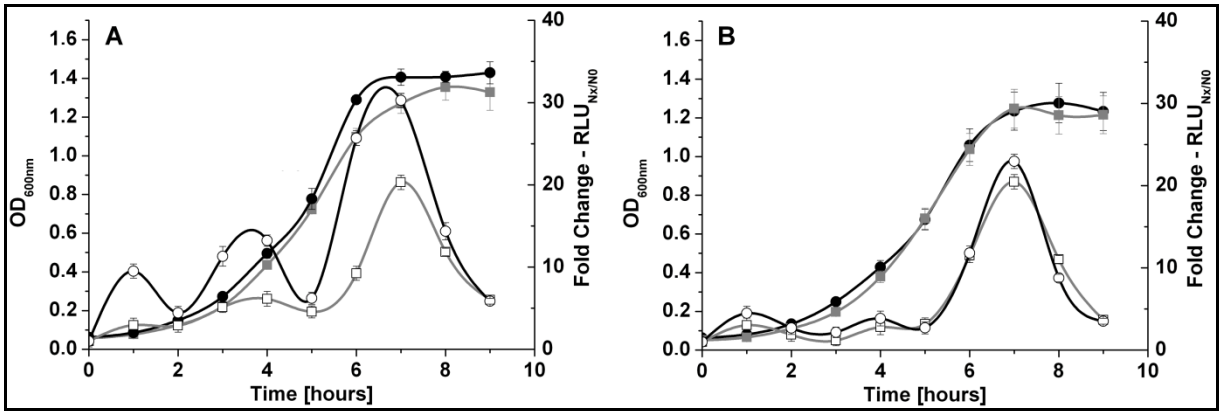

Figure 13. Promoter activity of *mleR* and *mleS* in the absence of malate.
Promoter activity of mleR and mleS during batch cultivation in BMS medium without malate under anaerobic conditions. A: Optical density and luciferase activity of both promoters in the wildtype background. B: Optical density and luciferase activity of both promoters in the ΔmleR background. Grey square, Optical density of strains carrying mleRp-luc; Black circle, Optical density of strains carrying mleSp-luc; Open square, RLU of strains carrying mleRp-luc; Open circle, RLU of strains carrying mleSp-luc

To rule out that this up-regulation was not due to post-exponential phase phenomena, we investigated the influence of the pH during the exponential growth phase in more detail (see below). However, in the wildtype the *mleS* promoter construct showed higher activity than in the Δ*mleR* knockout strain, indicating that MleR induces transcription even in the absence of the potential co-inducer L-malate. Accordingly, quantitative real time RT-PCR of RNA isolated from cells in the late exponential phase in the absence of L-malate showed a 3-fold induction of the genes *mleS* and *mleP* when comparing the wildtype to the Δ*mleR* mutant strain. An induction of *mleR* itself under these conditions was not observed (data not shown).

2.3.4 Regulation of the *mle* genes by pH and L-malate

During batch cultivation without addition of external L-malate, the highest luciferase activity for the *mle* promoters was observed during the transition to the stationary phase (see above). Addition of L-malate as free acid to the culture (end concentration of 25 mM), thereby lowering the pH to 5.6-6.2 (depending on the growth stage in BM medium), resulted in an immediate induction of activity (Figure 14).

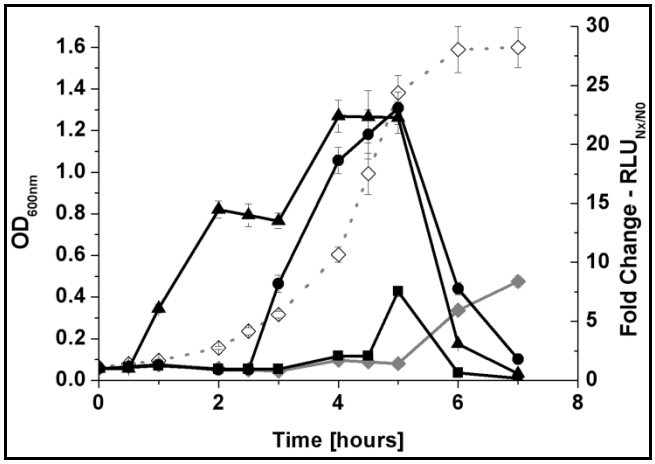

Figure 14. Promoter activity of *mleR* in the presence of malate.
Influence of L-malate (25 mM, not neutralized) on the promoter activity of wildtype S. mutans carrying mleRp-luc in BMS medium under anaerobic conditions. Open diamond, growth without malate; Grey diamond, RLU, no addition of L-malate; Triangle, RLU, addition of L-malate after 30 min; Circle, RLU, addition after 2.5 hours; Square, RLU, addition after 4.5 hours.

To determine if this effect was caused by the low pH or by L-malate, we further studied the influence of both parameters separately. After inoculation, cells were allowed to adapt for two hours to the medium. After addition of neutralized L-malate (25 mM final concentration) the pH of the cultures was adjusted with HCl to the desired values and samples for luciferase measurements were withdrawn in intervals of 30 min for two hours. Figure 15 summarizes the fold change values of promoter activity after two hours of measurement. Lowering the pH, without addition of malate, resulted in an increased activity of both promoters in the wildtype as well as in the *ΔmleR* background.

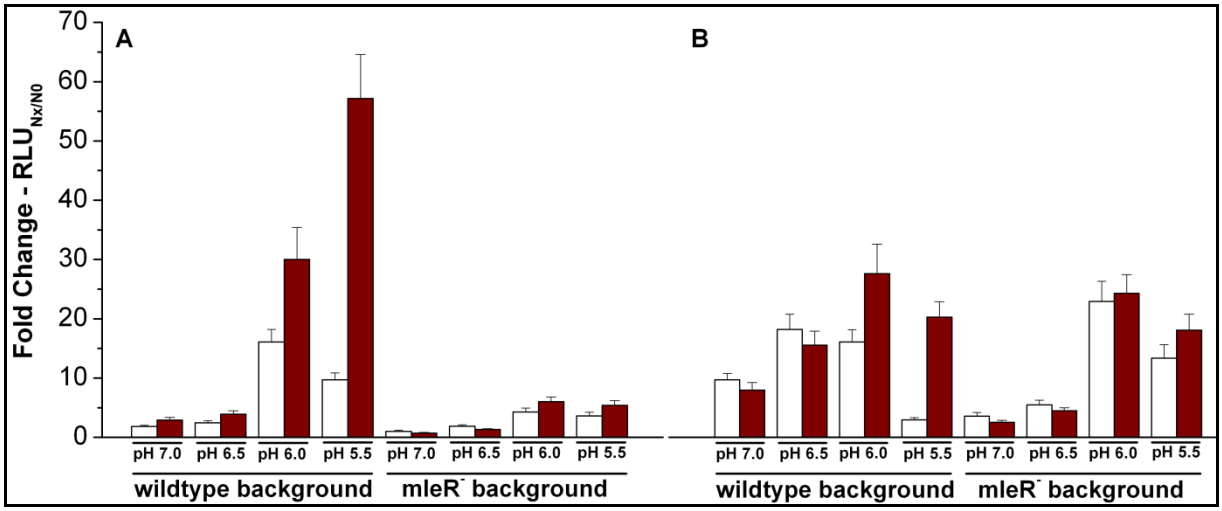

Figure 15. Influence of pH and L-malate on promoter activity of *mleR* and *mleS*.

Cells of wildtype and ΔmleR were cultivated in BMS under anaerobic conditions. Neutralized L-malate was added to the respective samples and the pH was adjusted to the desired values. A: Fold change of RLU after two hours of strains carrying mleSp-luc. Left, wildtype. Right, ΔmleR mutant . B: Fold change of RLU after two hours of strains carrying mleRp-luc. Left, wildtype. Right, ΔmleR mutant. White bars, no addition of L-malate; Red bars, addition of 25 mM L-malate.

These data clearly demonstrate that both promoters are acid inducible and that this behaviour was not caused by post-exponential phenomena. Furthermore, it shows that the influence of MleR is weak at neutral pH conditions. By contrast, the presence of L-malate at low pH significantly enhanced the activity of both promoters, but only in the presence of a functional copy of *mleR*. This allows four conclusions: (a) L-malate is the coinducer of MleR; (b) enhanced transcription in the presence of L-malate requires an acidic pH; (c) MleR positively regulates its target genes and furthermore (d) its own transcription. A positive auto-regulation would be a special feature, since most LTTR repress their own transcription. However, exceptions exist e.g. LrhA (Gibson and Silhavy 1999). However, no significant induction of *mleR* after two hours exposure to 25 mM free malic acid was observed using quantitative real time PCR (See below).

2.3.5 Quantitative real time PCR

The transcript levels of the genes SMU.135-140 were determined using quantitative real time RT-PCR. To this end, an early log phase culture of the wildtype was divided. To one part free malic acid (25 mM final concentration) was added, the other part remained untreated. RNA was sampled prior to splitting the culture and after two hours. All tested genes, except *mleR* itself, showed enhanced transcription in the presence of malic acid compared to time zero (Figure 16).

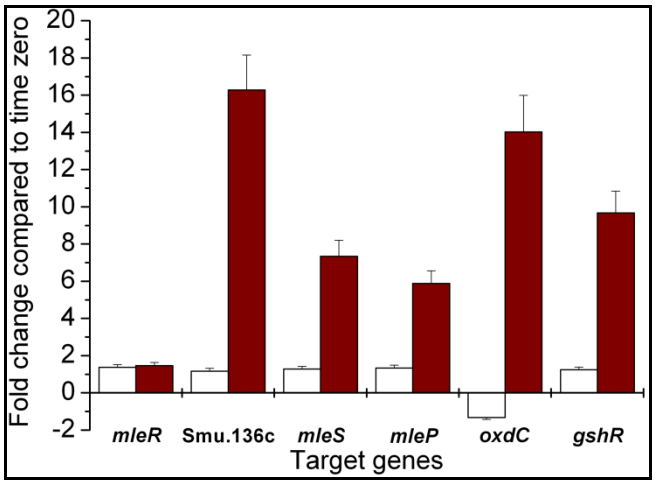

Figure 16. Induction of the *mle* locus by low pH and malate.
The transcription level was determined by quantitative real time RT-PCR of the genes SMU.135-140. Results are presented as fold change after a two hours treatment with 0 or 25 mM L-malate and compared to time zero. White bars, 0 mM free malic acid; Red bars, 25 mM free malic acid.

2.3.6 Influence of L-malate and MleR on growth

Since L-malate does not serve as a catabolite facilitating growth of *S. mutans* we were interested to see how energy gain and pH maintenance due to MLF affect its ability to grow in an acidic environment. To study this, we used BM medium supplemented with 1% (w/v) glucose (pH adjusted to 6.0) with or without supplementation of L-malate. In the absence of L-malate, there was no difference in growth of the wildtype and the Δ*mleR* mutant strain. Both strains entered the stationary phase after 6-7 hours at an external pH of about 4.2 and reached a final OD_{600} of about 0.41 (Figure 17 A). Inoculation of neutral BMG with this culture (pH 7.4) resulted in an optical density of ~ 1.0 for both strains, ensuring that the pH and not nutrient limitation were the determinant for entering the stationary phase at acidic conditions.

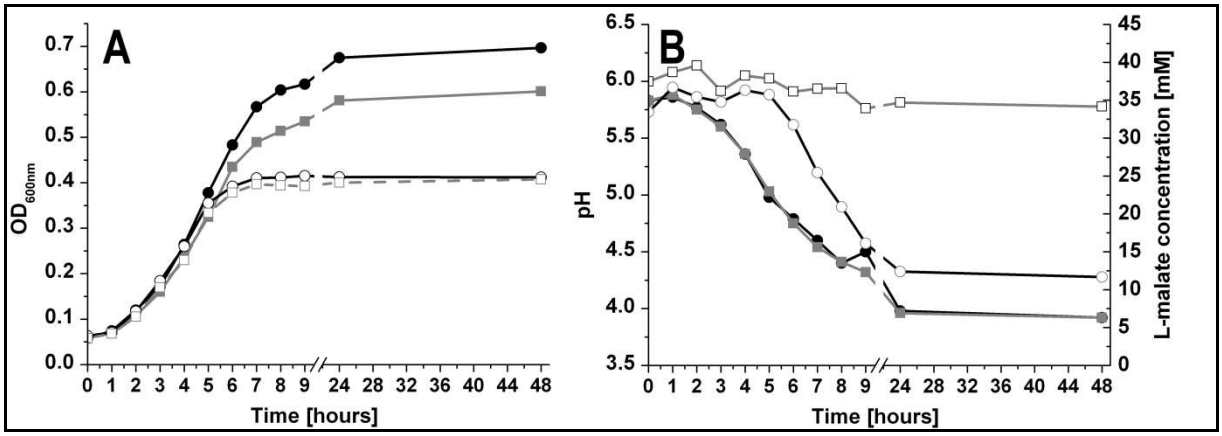

Figure 17. Influence of L-malate and *mleR* on the growth of *S. mutans*.
Cell were inoculated in acidified BMG (pH 6.0) medium under anaerobic conditions. A: Growth (OD600) of wildtype (black) and ΔmleR mutant (grey) in the absence (open symbols) or presence (filled symbols) of L-malate. B: pH and malate concentration of the supernatant of wildtype and ΔmleR mutant cultures grown in the presence of malate. Closed circle, pH of wildtype; Closed square, pH of the ΔmleR mutant; Open circle, malate concentration of wildtype; Open square, malate concentration of the ΔmleR mutant.

Addition of L-malate to the acidified culture medium facilitated pH maintenance and further growth of both cultures (Figure 17 A). The presence of L-malate resulted in a substantially higher optical density of the wild type compared to the *mleR* knockout strain. Both strains were capable of carrying out MLF, as monitored by the L-malate concentration in the supernatant (Figure 17 B), but the mutant to a much smaller degree than the wildtype. Further on significant internalisation/decarboxylation of L-malate started when the external pH dropped below 5, confirming the luciferase reporter data which had shown that the malolactic fermentation system is only activated at low pH.

2.3.7 Influence of L-malate and *mleR* on the ability of *S. mutans* to tolerate acid stress

Since MLF has been shown to facilitate pH maintenance (Sheng and Marquis 2007), we studied the contribution of MLF to acid tolerance in *S. mutans* (Figure 18). Control cells of wildtype and *ΔmleR* were grown in neutral THBY before being transferred to pH 3.1 without L-malate. Both strains showed no difference in the survival under these conditions (Figure 18). To determine the influence of malate and the *mleR* regulator on the response of *S. mutans* to a rapid pH shift, both the wildtype and the *mleR* mutant were grown in neutral THBY and then subjected to pH 3.1 in the presence of 25 mM malate. In both strains the number of surviving cells after 20 minutes was similar to the control (Figure 18). However, after 40 minutes the number of viable cells increased significantly compared to the control in the

wildtype. Thus, the genes for MLF were induced within this time period and the conversion of malate contributed to the aciduricity. Without a functional copy of *mleR*, the number of viable cells also increased after 40 minutes but to a much lesser extend compared to the wildtype. This again shows that a shift to an acidic pH is satisfactory to induce the MLF genes in the absence of *mleR*. When the *mle* genes were induced by low pH and L-malate in a preincubation step before transferring the cells to pH 3.1, an immediately increased viability was already seen 20 minutes after acid shock. Again, the wildtype exhibited a significantly enhanced survival compared to the *mleR* knockout mutant. The data show that the MLF genes are induced during the acid adaptation response but a functional copy of *mleR* in conjunction with its co-inducer L-malate is needed to achieve maximal expression.

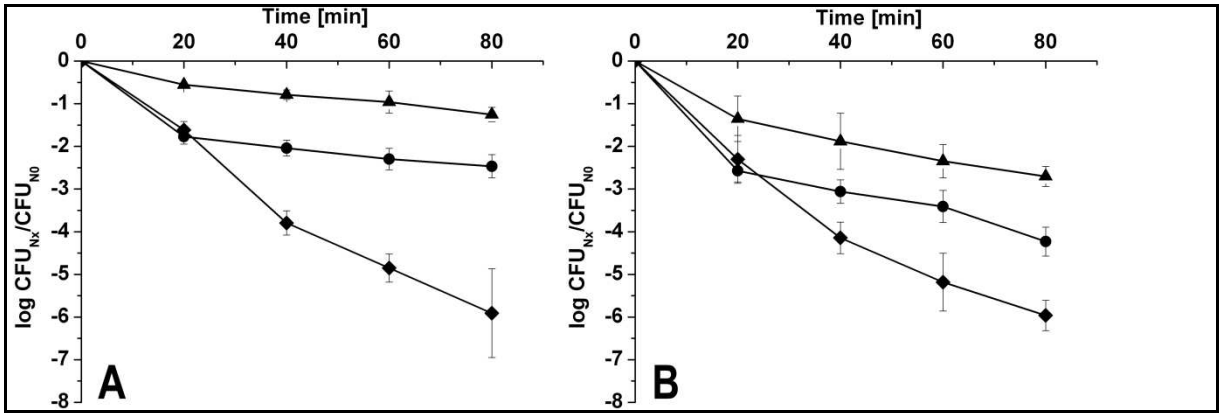

Figure 18. Acid tolerance assay.
Role of malate for the survival of S. mutans wildtype (A) and ΔmleR mutant (B) after acid stress. Diamond, control, cells were incubated in neutral THBY without malate and subjected to pH 3.1 without malate; Circle, cells were incubated in neutral THBY without malate and subjected to pH 3.1 with malate; Triangle, cells were incubated in acidified THBY with malate and subjected to pH 3.1 with malate.

Quantitative real time PCR showed an up-regulation of the adjacent gluthatione reductase upon the addition of 25 mM free malic acid (Figure 16). Therefore, we tested the capability of *S. mutans* to survive exposure to 0.2 (v/v) hydrogen peroxide after incubation of cells in acidified THBY and malate to induce this gene. However, no difference between wildtype and Δ*mleR* mutant was observed (data not shown).

2.4 **Discussion**

The aciduric capacity of *S. mutans* is one of the key elements of its virulence. Contributing mechanisms are increased activity of the F1F0-ATPase, changes in the membrane protein and

fatty acid composition, the induction of stress proteins and the production of alkaline metabolites (Griswold et al. 2006, Len et al. 2004b, Fozo and Quivey, Jr. 2004, Hasona et al. 2007). Extrusion of protons via the F1F0-ATPase consumes energy in the form of ATP. Hence, the yield of glycolytic activity and ATP production is diminished at low pH, *S. mutans* has to induce other pathways to supply enough energy. The conversion of L-malate to L-lactate and carbon dioxide during malolactic fermentation facilitates the maintenance of the ATP pool of the cell and supports the production of more alkaline metabolites. Therefore MLF directly contributes to the competitive fitness of *S. mutans* in the complex, multispecies environment of the dental plaque. Recently, Sheng and Marquis showed that cells of *S. mutans* UA159 possess MLF activity but no information about its regulation was available (Sheng and Marquis 2007). According to the information of MLF from *L. lactis* it was likely that the LTTR *mleR* adjacent to the MLF genes might be involved in their regulation.

Low pH is required for induction of MLF. A knockout of *mleR* significantly decreased MLF activity of *S. mutans* cells and thus confirmed its participation in the regulation of MLF. Applying promoter luciferase reporter constructs we showed that the regulation of the *mle* genes is much more complex than just being induced in the presence of MleR.
The luciferase fusion data and the acid killing profiles showed that the *mle* genes are activated within 30 minutes by acidic pH values, independently of MleR and malate. Therefore, the transcription of the *mle* genes is driven from acid inducible promoters and MLF is part of the early acid tolerance response. The EMSA experiments showed a clear interaction of MleR with malate, even under alkaline conditions. However, under neutral pH conditions no effect of malate on the transcription (using the luciferase reporters) was noticeable, suggesting that uptake of malate occurs only under low pH conditions. Indeed, Poolman *et al*. (Poolman et al. 1991) showed that in the presence of a pH gradient, membrane vesicles of *L. lactis* are able to take up L-malate with one proton or the monoanionic form of L-malate (MH^-). They conclude that a pH gradient stimulates indirectly a malate/lactate antiport, by affecting the L-lactate gradient or promotes directly electrogenic malate uptake, respectively. They showed that with decreasing pH, the pH gradient adjusted to the membrane potential or even exceeded it, which resulted in an increased uptake of added malate. Assuming a similar mechanism in *S. mutans* explains why malate under neutral pH conditions did not cause an induction of the *mle* genes. Since the uptake of malate is reduced in a neutral pH environment, the intracellular amount of malate is not sufficient to stimulate MleR and subsequent avoided a positive regulation.
MleR fully induces the MLF only at low pH, with malate acting as a coinducer. A similar mechanism was recently disclosed by Liu *et al*. for the agmatine deiminase system (Liu et al. 2009). They showed that its induction by AguR requires both low pH and agmatine. Using a linker scanning mutagenesis approach they were able to isolate mutant forms of AguR that lost their ability to activate transcription in response to pH, agmatine or both signals, respectively. They suggested that acidic conditions favoured binding of the ligand due to

conformational changes of the regulator protein. A similar mechanism may indeed also be true for MleR and L-malate.
In *S. mutans*, MLF is switched on at low pH in the complete absence of malate. This behavior might be adaptive since low pH and the availability of malate are often correlated in natural sources, e.g. fruits. Thus, it may be advantageous for *S. mutans* to induce the whole battery of acid tolerance responses if threatened by low pH in order to be prepared, since chances of encountering malate are usually high.

The *mle* locus. By RT-PCR we showed that the oxalate decarboxylase gene (*oxdC*) is co-transcribed with the *mleSP* genes. Since the reactions catalysed by MleS and OxdC are analogous it can be expected that decarboxylation of oxalate to formate also contributes to the aciduricity of *S. mutans*. However, no evidence for oxalate decarboxylation activity was found in *S. mutans* under the tested conditions, but extensive investigations were not carried out. Examination of the transcript levels of the wildtype in the presence of free malic acid using quantitative real time PCR showed co-transcription of *oxdC* with the *mle* genes and confirmed the results obtained with the luciferase reporter strains. The transcript level of *mleR* itself constituted an exception because it was not elevated. However, the result has to be interpreted cautiously since the reporter strains used here do not take into account the mRNA stability of *mleR*, which might represent another regulatory mechanism. Furthermore qPCR showed an induction of the adjacent gluthatione reductase, confirming that the responses to acidic and oxidative stress are overlapping in *S. mutans* (Svensater et al. 2000).

MleR binding sites. The electrophoretic mobility shift assays shown here revealed the presence of multiple binding sites for MleR in the DNA region within the translational start site of *mleR* and *mleS*. LysR type transcriptional regulators (LTTR) are generally regarded to be active as tetramers, therefore they are known to interact with several binding sites at their promoter region(s). The (auto)-regulatory binding site is favoured by the apo-form, whereas the (target)-activation site is occupied once the co-inducer is bound to the protein. However, the presence of the co-inducer affects the affinity to each binding site, influences DNA bending and subsequently protein-protein interactions (Maddocks and Oyston 2008, Tropel and van, Jr. 2004).
The addition of L-malate changed the retardation pattern for some of the applied DNA fragments. Since the transcription of *mleR* and *mleS* was shown to be induced equally by a pH shift and L-malate using the luciferase reporter strains, a similar retardation behaviour in the EMSA for both upstream DNA fragments would have been expected.
Surprisingly, only the IGS upstream of *mleS* showed a different pattern in the presence of malate, whereas the IGS upstream of *mleR* even showed a weaker retardation. Due to the basic pI of the MleR protein, we were not able to carry out EMSA under physiological pH conditions which might negatively influence the binding affinity of the protein. The presence

of at least two binding sites for MleR within the coding region of SMU.136c suggests a complex regulatory mechanism, which has to be elucidated further by means of DNase footprinting and mutagenesis.

Conclusion

In summary, we showed that the *mle* genes including *oxdC* are under the control of acid inducible promoters and that they are induced within the first 30 minutes upon acid shock. Therefore they are part of the early acid tolerance response in *S. mutans*, which is induced within 30 minutes after acidification (Svensater et al. 1997). Further enhancement of their transcription can be obtained by MleR and L-malate in an acidic environment. The use of gel retardation assays showed the presence of multiple binding sites for MleR, even in the coding sequence of another gene, suggesting a complex regulatory mechanism. We clearly showed that the presence of L-malate contributed strongly to the survival of *S. mutans* under low pH conditions. MLF is one of the strategies aciduric bacteria have evolved to cope with low pH and to compete with other bacteria in dental plaque. *S. mutans* is able to carry out MLF under more acidic conditions than other *Streptococci* (Sheng and Marquis 2007), thus emphasizing the dominant role of *S. mutans* in the oral cavity.

Acknowledgement

We would like to thank Andreas Podbielski for providing the pFW5 plasmid and Holger Lössner for providing the pHL222 plasmid.

CHAPTER III

Analysis of the *luxS* mutation in *Streptococcus mutans* UA159 - effects of genetical, physiological and chemical complementation

André Lemme, Helena Sztajer, Irene Wagner-Döbler

Helmholtz-Centre for Infection Research, Research Group Microbial Communication, Inhoffenstr. 7, D-38124 Braunschweig.

3 Chapter - III Analysis of the *luxS* mutation in *Streptococcus mutans* UA159 - effects of genetical, physiological and chemical complementation

3.1 Abstract

The dual role of the *luxS* gene, which is involved in a central activated methyl cycle (AMC) and in the synthesis of the autoinducer-2 (AI-2) signaling molecule, makes an analysis difficult since observed phenotypes obtained from a mutation in the *luxS* gene can result from defects in metabolism or in signalling. The aim of this study was to complement a *luxS* mutant of *Streptococcus mutans* UA159 genetically, physiologically by heterologous expression of the SAH-hydrolase (*sahH*) – an alternative enzyme of the AMC - and chemically by the addition of chemically synthesized AI-2. During complementation, we identified secondary mutations in a previously published *luxS* mutant. Construction of a new *luxS* deletion strain and analysis of its phenotype revealed that LuxS affected the glutamate and sulfur metabolism but had no influence on the main virulence traits of *S. mutans* (biofilm formation, aciduricity, and mutacin production). Expression of *luxS* and its upstream sequence from a plasmid did not restore these effects but resulted in a severe impairment of all tested virulence phenotypes. Introduction of *sahH* restored the changes in glutamate and sulfur metabolism beyond wildtype levels, showing that the changes in glutamate and sulfur metabolism were caused by disruption of the AMC and not by secondary mutations. Moreover, expression of *sahH* resulted in differences in the aciduric and biofilm formation capacities of *S. mutans*. Addition of AI-2 at various concentrations had no influence on the tested phenotypes and on gene expression determined with a microarray.
Our data show that deletion of *luxS* caused differences in gene expression affecting the glutamate and sulfur metabolism but that these changes did not influence the main virulence traits of *S. mutans*. Furthermore, our data demonstrate that AI-2 does not play a role as a signaling molecule for *S. mutans* under the conditions used in this study.

3.2 Introduction

The discovery that autoinducer-2 (AI-2) is involved in the complex, multilayered quorum sensing system in *Vibrio sp.* and the wide spread distribution of the cognate synthase LuxS

(Sun et al. 2004) lead to intensive investigations carried out in many bacteria, including members of the oral cavity, like *Streptococcus mutans* (Federle 2009, Turovskiy et al. 2007). The AI-2 producing enzyme LuxS is incorporated in the activated methyl cycle (AMC) and thus an integral part of the central metabolism of the cell. During various methyl transferase reactions *S*-adenosylmethionine (SAM) is converted to *S*-adenosylhomocysteine (SAH), which is a potent feedback inhibitor of SAM-dependent methylations, due to its structural similarities (Winzer et al. 2002). SAH is further converted by the Pfs enzyme and yields *S*-ribosylhomocysteine (SRH) and adenine. SRH is cleaved by the LuxS enzyme to homocysteine and (*S*)-4,5-dihydroxy-2,3-pentanedione (AI-2), which spontaneously rearranges to various furanone derivates, which are collectively termed AI-2 (Chen et al. 2002, Miller et al. 2004, Schauder et al. 2001). However, all eukaryotes, and archaebacteria, and some eubacteria use the enzyme SAH-hydrolase (SahH) for the detoxification of SAH in an one step mechanism, yielding homocysteine and adenosine, thus by-passing the production of AI-2 (Sun et al. 2004).

Due to the dual role of the LuxS enzyme in the central metabolism as well as for AI-2 production, results from analysing Δ*luxS* mutants have to be interpreted cautiously, since it cannot be distinguished if observed phenotypes arose from defects in metabolism or AI-2 signalling, respectively. The majority of studies, dealing with the *luxS* mutation in *S. mutans*, focussed on changes in its main virulence traits, e.g. biofilm formation or resistance to various stresses.

The study from Sztajer *et al*. was the only one trying to separate *luxS* and AI-2 dependent regulatory circuits on the level of transcription in *S. mutans* (Sztajer et al. 2008). However, despite the extensive use of microarray analysis and subsequent investigation of various potential AI-2 targets, no mechanistic roles for AI-2 could be revealed and confirmed in *S. mutans* so far.

The aim of this study was to introduce a SAH-hydrolase gene into a *luxS* mutant of *S. mutans* UA159 to restore the disrupted metabolic function of the AMC physiologically and to reveal the role of AI-2 in this strain by addition of pure chemically synthesized AI-2.

During the investigation we noticed the presence of secondary mutations in the *luxS* mutant of *S. mutans* U159 published previously (Sztajer et al. 2008). Since *luxS* is embedded in an important central metabolic cycle and its deletion is often associated with pleiotropic phenotypes, suppressor mutations may occur frequently to compensate the defect of the AMC. Similar observations have been reported for *Staphylococcus aureus* and *Lactobacillus rhamnosus* GG (Doherty et al. 2006, Lebeer et al. 2007). The genuine *L. rhamnosus* Δ*luxS* mutant showed attenuated biofilm formation and slower growth under conditions of cysteine and vitamin depletion. Both traits could be restored by either genetic complementation or addition of cysteine and vitamins. Interestingly, the strain containing secondary mutations showed no growth defects but attenuated biofilm formation, which could not be restored

genetically or by the addition of nutrients (Lebeer et al. 2007, Lebeer et al. 2008). Currently, the location of these secondary mutations is not known.
We show here that the previously reported defect in biofilm formation of the *luxS* mutant of *S. mutans* UA159 (Sztajer et al. 2008) was not due to disruption of the *luxS* gene but rather the result of secondary mutations. We report here the construction of a new *luxS* mutant in *S. mutans* UA159 and its genetical, physiological and chemical complementation. Analysis of the main virulence traits of *S. mutans* and a microarray analysis were carried out for the *luxS* mutant and its complementation constructs. To distinguish between metabolic effects due to disruption of the AMC and AI-2 signalling, the *luxS* mutant strain expressing the SAH-hydrolase was additionally complemented with AI-2.

3.3 **Results**

3.3.1 Secondary mutations in a *luxS* mutant.

Since the occurrence of secondary mutations in *luxS* mutants of other strains has been reported (Doherty et al. 2006, Lebeer et al. 2007), we wanted to exclude this possibility and constructed a plasmid for genetic complementation of the previously published $luxS_1$ mutant. Therefore, we cloned the 500 bp upstream of the *luxS* gene and its complete coding sequence into the shuttle vector pDL278 yielding pDLluxS.

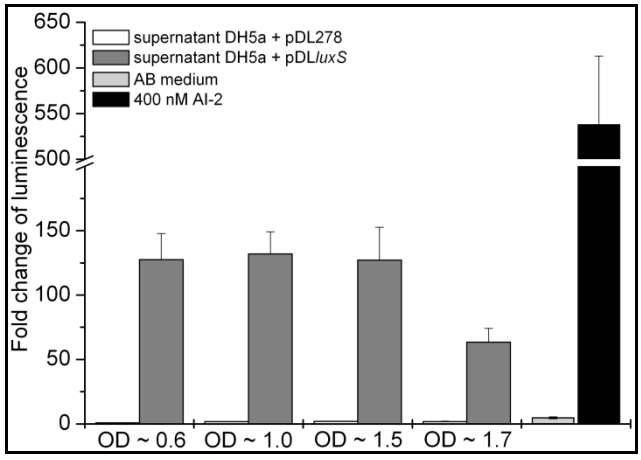

Figure 19. *Vibrio harveyi* bioassay to monitor AI-2 activity.
Culture supernatants of *E. coli* DH5α carrying pDL278 or pDLluxS of different optical densities were tested for AI-2 activity using the *V. harveyi* bioassay. Pure chemically synthesized AI-2 or sterile AB medium were used as positive or negative control, respectively. Means and standard deviation of duplicate experiments are shown.

To verify that a functional *luxS* gene was present in this plasmid, the supernatant of *E. coli* DH5alpha carrying pDLluxS was successfully tested for autoinducer-2 activity using the *Vibrio harveyi* bioassay (Figure 19). We had previously reported a severe defect in sucrose dependent biofilm formation of the *luxS*$_1$ mutant of *S. mutans* UA159, regardless of the used media (Sztajer et al. 2008). Moreover, the *luxS*$_1$ mutant displayed a smooth colony morphology in contrast to the rough colonies of the UA159 wildtype strain.
Despite the presence of the pDLluxS plasmid in the *luxS*$_1$ mutant of *S. mutans* (verified by PCR), a phenotypic reversal with respect to its ability to form biofilms (data not shown) and its colony morphology was not achieved (Figure 20), indicating that suppressor mutations had occurred in the Δ*luxS*$_1$ strain. We therefore repeated the deletion of the *luxS* gene using the same PCR mutagenesis approach described in Sztajer *et al*. (Sztajer et al. 2008) to obtain *S. mutans* Δ*luxS*$_2$. Deletion of *luxS* was confirmed by sequencing of the *luxS* locus and showed that replacement of *luxS* with the erythromycin cassette occurred in the same way as in Δ*luxS*$_1$.

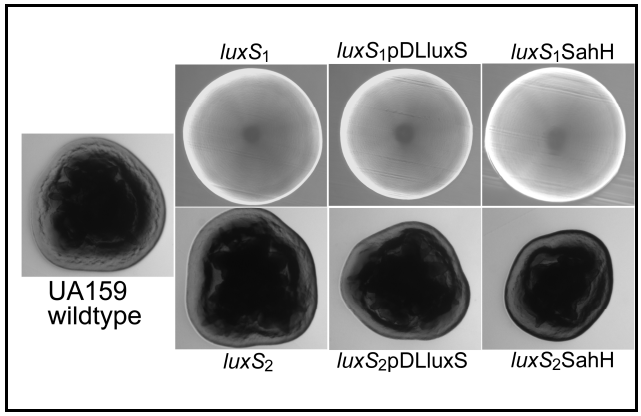

Figure 20. Colony morphology of *S. mutans* UA159 and its derivates.
Upper panel, colony morphologies of the *luxS*$_1$ mutant from Sztajer *et al*. (Sztajer et al. 2008) and its complementation strains. Lower panel, the *luxS*$_2$ mutant strain constructed for this study and its complementation strains. Between both panels, the UA159 wildtype strain is shown.

Determination of the colony morphology clearly showed that the secondary mutations, responsible for the smooth morphology in Δ*luxS*$_1$, were absent in the new construct Δ*luxS*$_2$ since this strain exhibited the same morphology as the wildtype strain (Figure 20). Lebeer *et al*. reported that prolonged incubation if the *L. rhamnosus* GG *luxS* mutant resulted in a frequent occurrence of secondary mutations (Lebeer et al. 2007). However, prolonged incubation and re-inoculation over several passages of the newly constructed *luxS*$_2$ mutant did not yield clones with the smooth colony morphology and we currently do not know the origin of the secondary mutations in the *luxS*$_1$ mutant. The *luxS*$_2$ mutant was used for further analysis and as a host for the integration of the SAH-hydrolase.

3.3.2 Introduction of the SAH-hydrolase into both *luxS* mutant strains and the wildtype

For expression of the *sahH* gene we decided to integrate this gene into the chromosome in place of the erythromycin cassette that had previously been used to replace part of the *luxS* gene. As source for the *sahH* gene we decided to use DNA of the soil bacterium *Acinetobacter baylyi* ADP1 because it has a low GC-content similar to *S. mutans*. To drive expression of the *sahH* gene we cloned the strong constitutive promoter (P*aad9* of the spectinomycin cassette (*aad9*)), including its own ribosomal binding site, from the pFW5 vector in front of the *sahH* gene. To select for positive clones after integration into the chromosome both fragments (P*aad9* and *sahH*) were cloned next to the spectinomycin cassette (*aad9*) of the pFW5 vector having the same orientation (Figure 21). To facilitate homologous recombination the upstream and downstream regions (~1000 bp), flanking the *luxS* locus, were amplified and cloned adjacent to the P*aad*9-*sahH-aad9* fragment into the pFW5 vector. After obtaining a successful integration, this construct would replace the erythromycin cassette by integrating the P*aad*9-*sahH-aad9* fragment in the same orientation as the erythromycin cassette or as the *luxS* gene were present before (Figure 21).

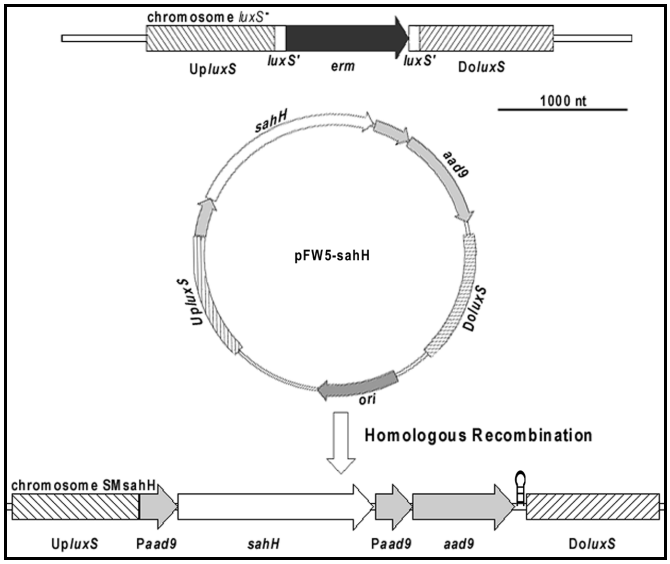

Figure 21. Integration scheme of pFW5-sahH plasmid into the *luxS* locus.

The *luxS* gene was replaced with an erythromycin cassette, which was further replaced by the *sahH* gene linked to a spectinomycin cassette. The lollypop symbol indicates a rho-independent terminator signal which is located downstream of the spectinomycin cassette (*aad9*). Double homologous recombination results in strain $\Delta luxS_2$SahH carrying in the chromosome.

For an additional confirmation of the secondary mutations in $\Delta luxS_1$ we transformed the pFW5-SahH plasmid into the wildtype, $\Delta luxS_1$ and $\Delta luxS_2$ strain, respectively.

Sequencing of spectinomycin resistant colonies confirmed the correct integration of this construct in all three strains. Consequently all three obtained strains had the same genotype considering the *luxS* locus, having the *luxS*/erythromycin gene replaced by the SAH-hydrolase gene linked to the spectinomycin cassette. However, the former Δ*luxS*$_1$ strain still showed the smooth colony morphology as before, confirming the presence of other mutations than that of *luxS*. Contradictory, we did not observe a different colony morphology by deletion of *luxS* alone (Δ*luxS*$_2$) or by simultaneous introduction of the *sahH-aad9* genes (Δ*luxS*$_2$SahH) (Figure 20).

3.3.3 Analysis of growth and virulence factors of the wildtype and the different complementation strains.

Growth in different media

To assess the role of the *luxS* mutation and its complementation constructs for growth, we measured the growth of WT, Δ*luxS*$_2$, Δ*luxS*$_2$pDLluxS and Δ*luxS*$_2$SahH in complex (BHI and THBY) and chemically defined (CDMG) media. Growth of all four strains was similar in complex media (data not shown). However, in chemically defined minimal media, the strains Δ*luxS*$_2$pDLluxS and Δ*luxS*$_2$SahH showed an extended lag phase, a slower growth rate and a reduced final optical density compared to the wildtype strain. There was no difference in growth between the wildtype strain and the Δ*luxS*$_2$ mutant (Figure 22).The additional metabolic burden of the strain Δ*luxS*$_2$pDLluxS carrying pDLluxS (this plasmid is maintained at about 20-30 copies per cell (Burne et al. 1999) plasmid and the expression of *sahH,* and the spectinomycin cassette in strain Δ*luxS*$_2$SahH, respectively, apparently have reduced the growth capacities in minimal media. In *S. aureus* it has been shown that the metabolic influence of LuxS on growth will become important under conditions of limitation for methionine precursors (Doherty et al. 2006) Since the *luxS* mutation had no influence on growth even in minimal media we conclude that (i) recycling of SAH was not necessary, since the pool of methionine or its precursors in the media was satisfactory and not growth limiting and (ii) conversion of SAH to SRH by the Pfs enzyme was sufficient to eliminate toxic effects of SAH.

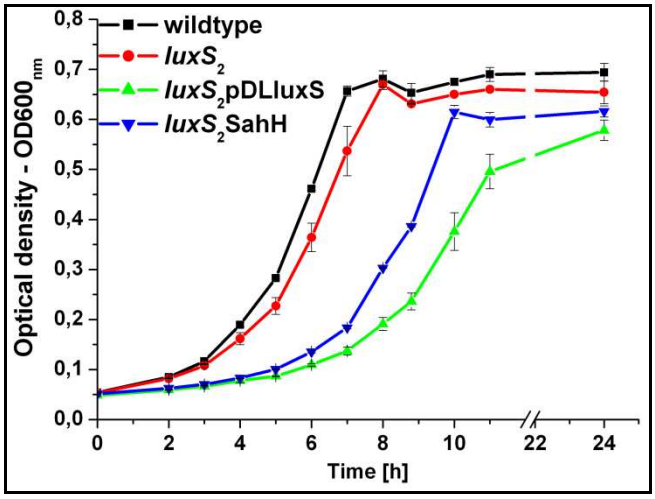

Figure 22. Influence of the *luxS* deletion and its genetical and physiological complementation on growth in CDM media.

Data are the means of duplicate experiments, and error bars indicate standard deviations.

Acid tolerance

Since aciduricity is one of the main virulence factors of *S. mutans*, we were interested to know how deletion of *luxS* and expression of *sahH* would influence this virulence determinant. In contrast to a previous study from Wen and Burne (Wen and Burne 2004), reporting a reduced acid tolerance of the *S. mutans* UA159 *luxS* mutant compared to the wildtype, we did not observe differences between the wildtype strain and the *luxS*$_2$ mutant (Figure 23). Maintenance of pDLluxS in Δ*luxS*$_2$ reduced the capacity to withstand acid stress, suggesting that either an increased expression of *luxS* due to the elevated copy number or the metabolic burden of the plasmid decreased the ability to cope with acid stress. By contrast, expression of the *sahH* gene in the absence of *luxS* enhanced the survival under acidic conditions, indicating that expression of *sahH* influences the metabolism of the *luxS*$_2$ mutant in a matter that affected its acid tolerance. The influence of autoinducer-2 was not tested for this virulence trait.

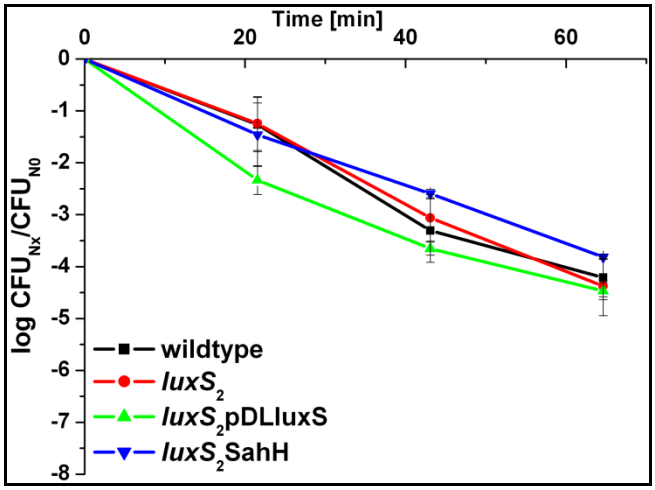

Figure 23. Capability of *S. mutans* and its derivates to withstand acid stress.
Data are the means of triplicate experiments, and error bars indicate standard deviations.

Genetic competence

Since competence development in *S. mutans* can be triggered by the quorum sensing signalling molecule CSP (competence stimulating peptide), we tested if AI-2 is able to influence the transformability of strain Δ*luxS*$_2$SahH. Transformation frequencies of Δ*luxS*$_2$SahH were similar to the wildtype, in the presence and absence of CSP (data not shown), indicating that the lack of AI-2 due to *luxS* deletion has no effect of CSP dependent and independent competence. Addition of AI-2 (10 nM, 2 µM and 25 µM), did not affect the transformation rate of wildtype and Δ*luxS*$_2$SahH (data not shown), showing that there is no crosstalk between CSP and AI-2 in competence development (CSP and AI-2 were added at the same time to the cultures).

Production of mutacin IV and V

Lack of LuxS was shown to abolish production of mutacin I but had no effect on production of mutacin IV in strain UA140 (Merritt et al. 2005). Strain UA159 lacks mutacin I but contains mutacin V, which is not present in strain UA140. The genome of *S. mutans* UA159 encodes for several putative mutacins but so far only mutacin IV and V have been shown to exhibit antibacterial activity against a panel of various indicator strains (Hale et al. 2005).
To determine the impact of the *luxS* mutation and its complementation strains on the major mutacins of *S. mutans* UA159, we carried out overlay assays using indicator strains for mutacin IV and V. We did not observe altered production of both mutacins in the absence of *luxS*. Maintenance of pDLluxS in the *luxS*$_2$ mutant reduced significantly the production of mutacin IV and V, respectively (Figure 24). To rule out the influence of the pDL278 vector,

we transformed pDL278 (empty vector) and pDLluxS into the wildtype and pDL278 into the *luxS*$_2$ mutant strain. Maintenance of pDLluxS in the wildtype confirmed the result of Δ*luxS*$_2$pDLluxS, since production of mutacin IV and V was abolished. The empty vector control in the wildtype and Δ*luxS*$_2$ showed no influence on the production of both mutacins, suggesting that an increased amount of LuxS interferes with the regulation of mutacin IV and V. Expression of *sahH* in the Δ*luxS*$_2$ background showed no effect on the production of both mutacins.
Addition of AI-2 to all strains in concentrations ranging from 10 nM to 50 µM had no influence (data not shown), indicating that AI-2 does not play a role for mutacin regulation in *S. mutans* UA159.

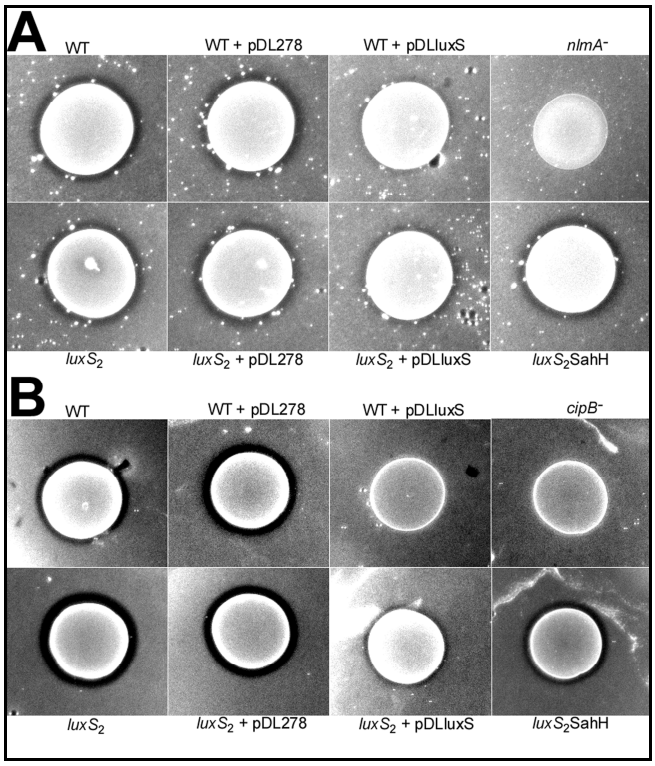

Figure 24. Overlay assay to visualize the production of mutacin IV and V of *S. mutans* and its derivates.
A: Overlay assay to detect the production of mutacin IV using *S. sanguis*. B: Overlay assay to detect the production of mutacin V using *L. lactis* 4067. Deletion mutants for mutacin IV (nlmA-) and mutacin V (cipB-) served as control. Twenty microliters of washed S. mutans overnight culture was spotted on an agar plate, dried for 20 minutes and afterwards overlay with the indicator strain. The black halo surrounding the spot shows the zone of growth inhibition of the indicator strain

Analysis of biofilm formation

Since the defects in biofilm formation reported previously (Sztajer et al. 2008) were not due to the *luxS* mutation, we analysed again the capability of Δ*luxS*$_2$ to form biofilms. In addition,

we supplemented the Δ*luxS*$_2$SahH strain with chemically synthesised AI-2 to determine the impact of AI-2 on biofilm formation. To this end, we added AI-2 into the biofilm forming media at 10 nM and 25 µM final concentration. The biomass was quantified using crystal violet staining (Figure 25).

Biofilm development of the wildtype showed that the biomass increased up to 8 hours post inoculation. Prolonged incubation up to 48 hours did not further affect the amount of biomass in the wildtype. The *luxS*$_2$ mutant achieved its highest biomass already 4 hours after inoculation. However, the final biomass of the *luxS*$_2$ mutant was similar to that of the wildtype.

The strain Δ*luxS*$_2$pDLluxS also displayed a faster biofilm growth than the wildtype, which resulted in more biomass within the first hours of growth. However, the final yield in biofilm biomass was lower than that of the wildtype and the *luxS*$_2$ mutant indicating that increased amounts of LuxS due to the higher copy number altered the biofilm formation capacities.

The biofilm formation of the strain Δ*luxS*$_2$SahH was similar to that of the wildtype. Addition of 10 nM and 25 µM of synthetic AI-2 to Δ*luxS*$_2$SahH had no influence on the time course of biofilm growth or the final amount of biomass.

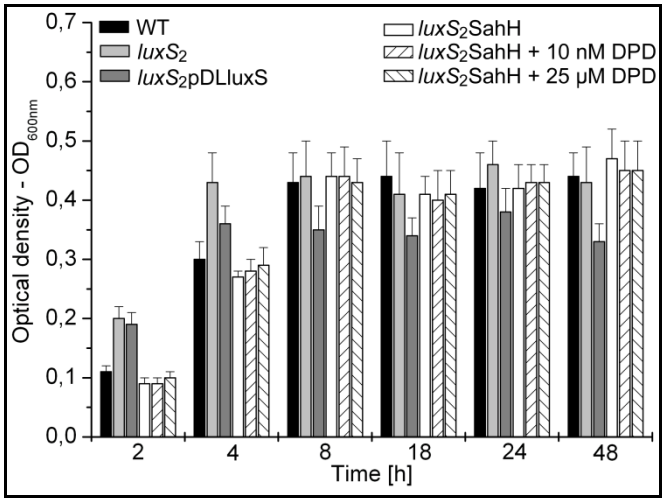

Figure 25. Time course of sucrose dependent biofilm formation.

Biofilms were grown for the designated time periods and the biomass was quantified using crystal violet staining. Data are the means of triplicate experiments, and error bars indicate standard deviations.

In order to determine structural differences in more detail, we investigated biofilm growth using confocal laser scanning microscopy (Figure. 26).

After 8 hours of incubation the microcolonies of the wildtype were slightly larger than those of the *luxS*$_2$ mutant. However, the overall structure and coverage of the biofilm on the substratum were similar for both strains. By contrast, strain Δ*luxS*$_2$pDLluxS was not able to

form larger microcolonies and also the height of the biofilm was reduced compared to the wildtype and the *luxS*$_2$ mutant. These structural changes are in accordance with the observed reduction in the biomass using the crystal violet staining procedure.

To deterime the influence of the pDL278 vector, we visualized biofilm formation of Δ*luxS*$_2$ carrying either the vector control pDL278 or the complementation plasmid pDLluxS by CLSM after 18 hours of incubation. No significant changes in the overall structure became apparent with the vector control, indicating that the observed effects were due to the cloned *luxS* gene and cannot be assigned to the presence of the host vector or incubation in the presence of spectinomycin. (Figure 27).

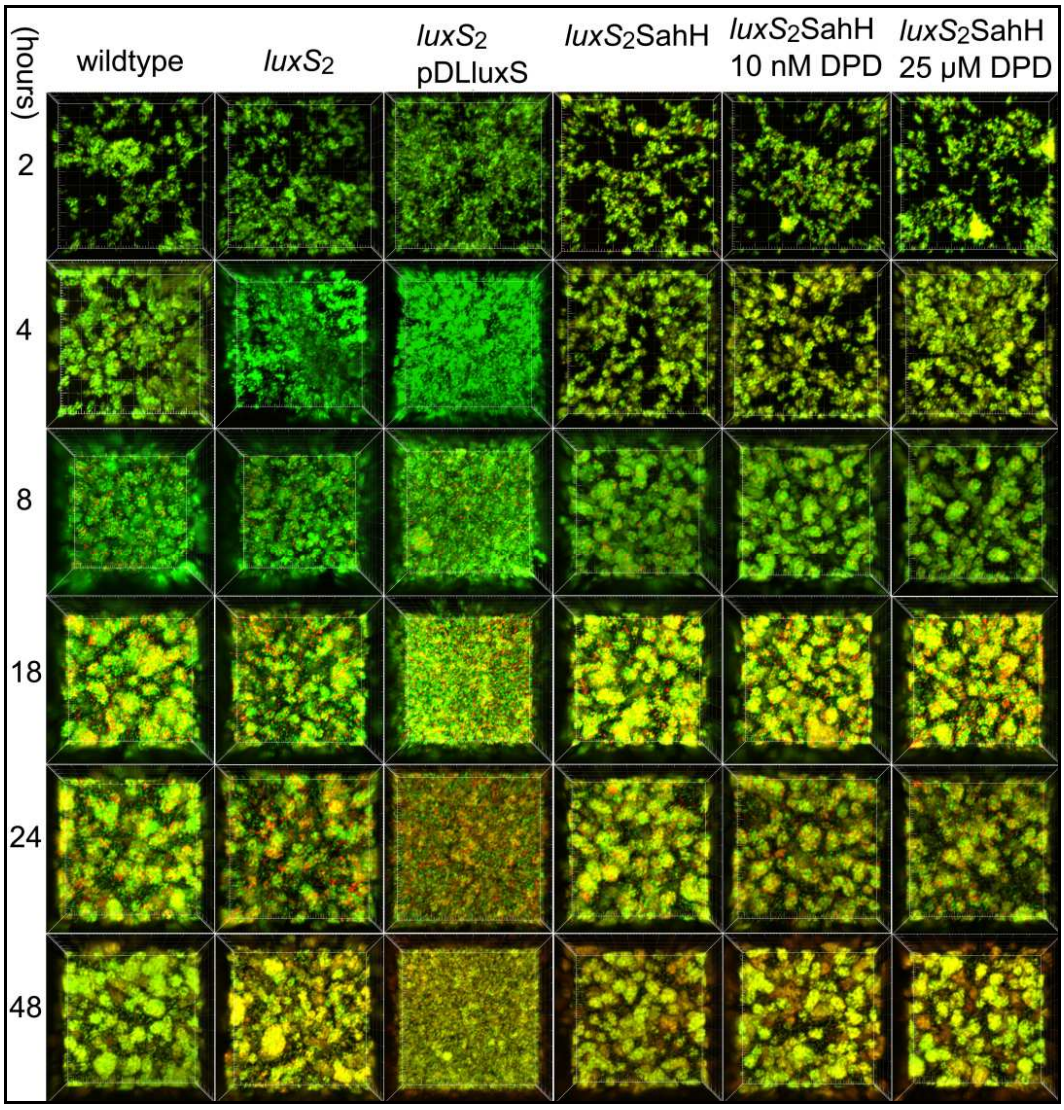

Figure 26. CLSM images of biofilm formation in BHIS.

The panels show the overall biofilm structure as top view at the designated time points. Biofilms were stained with the LIVE/DEAD *Bac*Light fluorescence dye. Dead cells are stained red, and live cells are stained green. The pictures show the overlay of the red and green channel, thus membrane damaged cells appear more yellow.

Strain Δ*luxS*$_2$SahH formed a biofilm having a different structure than that of the wildtype or the *luxS*$_2$ mutant. Microcolonies were formed earlier and were much more dense and compact. In addition, the cell layers between the microcolonies showed a reduced height compared to that of the wildtype. Using the BacLight viability stain for visualisation of membrane damage revealed an interesting observation within the first hours of biofilm formation. Strain Δ*luxS*$_2$SahH exhibited a higher number of dead/damaged cells (shown by the yellow color in Figure 26) at 2 and 4 h, which could not be associated with starvation, since the biomass was still increasing and the majority of cells in the other strains was exclusively stained with the green dye syto9. Addition of synthetic AI-2 at concentrations of 10 nM and 25 µM had no influence on the biofilm structure of Δ*luxS*$_2$SahH, assuming that AI-2 signalling does not play a role in mono-species biofilm formation of *S. mutans*.

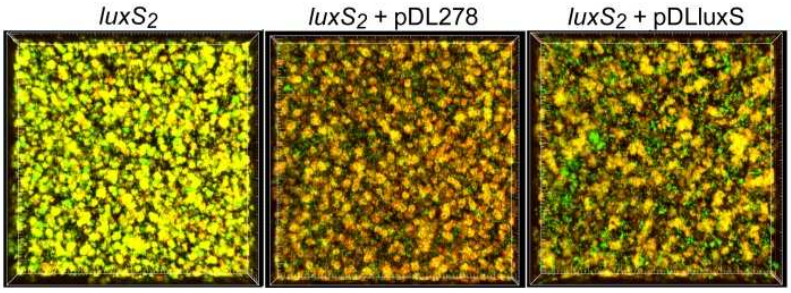

Figure 27. Influence of pDL278 on biofilm formation.
Images show the overall biofilm structure as top view after 18 h post inoculation. Biofilms were stained with the LIVE/DEAD *Bac*Light fluorescence dye. Dead cells were stained red, and live cells were stained green. The pictures show the overlay of the red and green channel, thus membrane damaged cells appear more yellow.

3.3.4 Biofilm transcriptome analysis

The survival of *S. mutans* in the oral cavity depends on the formation of biofilms on the tooth surface. The biofilm mode of growth represents an ideal environment for accumulation of autoinducers, due to close cell-cell contact and diffusion limitations within the biofilm matrix. Therefore we decided to carry out a transcriptome analysis of the different *S. mutans* strains grown as biofilms in the presence of sucrose. In addition, strain Δ*luxS*$_2$SahH was supplemented with two concentrations of synthetic AI-2 (10 nM and 25 µM). The microarray analysis was carried out 8 hours post inoculation, since at this time the biofilms were in the maturation phase.

General overview

Using the wildtype transcriptome as a reference, 37 genes were differentially expressed (fold change ± 1.7, P<0.05) in the *luxS*$_2$ mutant, confirming that deletion of *luxS* caused only minor changes in *S. mutans* UA159, congruent with the results obtained from comparing the growth, aciduricity, mutacin production and biofilm formation, which were not or only slightly affected by the deletion of *luxS*. By contrast, strain Δ*luxS*$_2$pDLluxS, showed large differences in its phenotypes compared to the wildtype or its parental strain Δ*luxS*$_2$. Accordingly, we obtained a huge number (340 genes) of differentially expressed genes comparing its transcriptome to that of the wildtype. Comparison of the transcriptome of Δ*luxS*$_2$SahH to the wildtype yielded 136 differentially expressed genes. Addition of 10 nM AI-2 to strain Δ*luxS*$_2$SahH resulted in 127 changed genes, whereas addition of 25 µM AI-2 resulted in 116 differentially expressed genes compared to wildtype transcriptome. Since it had to be expected that the presence of AI-2 altered the expression of some genes, putative AI-2 influenced genes were examined in more detail without restriction of a fold change of ± 1.7 but retaining the P-value. However, the trend of expression for all genes was similar to the strain Δ*luxS*$_2$SahH containing no AI-2. Thus in the following description, the transcription data of Δ*luxS*$_2$SahH and both chemically complemented strains are combined. Assignment of the genes to metabolic pathways was carried out manually according to their annotation and is presented in figures 28-30. A selection of differentially expressed genes of all strains is presented in table 3 and their roles are explained below. For better comparison the fold change values of all transcriptome experiments are presented, even when the difference compared to the wildtype was below the used cutoff (fold change ± 1.7, P < 0.05). For detailed information on all differentially expressed genes see supplementary tables 1-3. To confirm the microarray data, the transcription of six genes was determined by quantitative real time PCR and the results are presented in supplementary table 4.

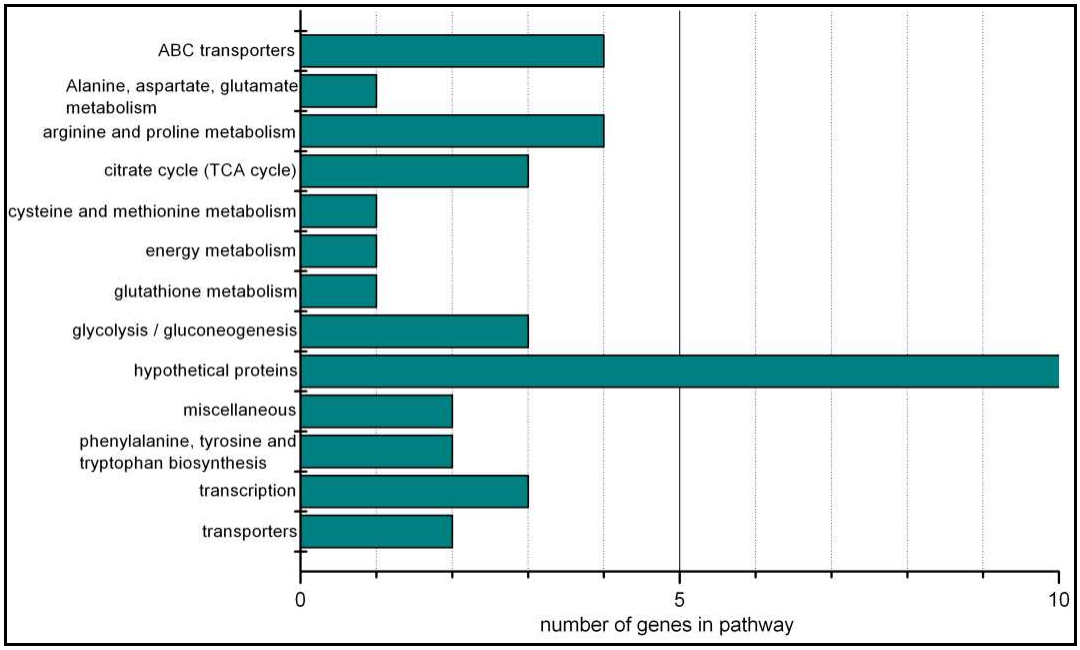

Figure 28. Assignment of differentially expressed genes into metabolic pathways.

Differentially expressed genes of the *luxS*$_2$ mutant, compared to wildtype, were assigned manually into metabolic pathways according to their annotation. From 37 genes, 17 were down regulated and 20 were up-regulated.

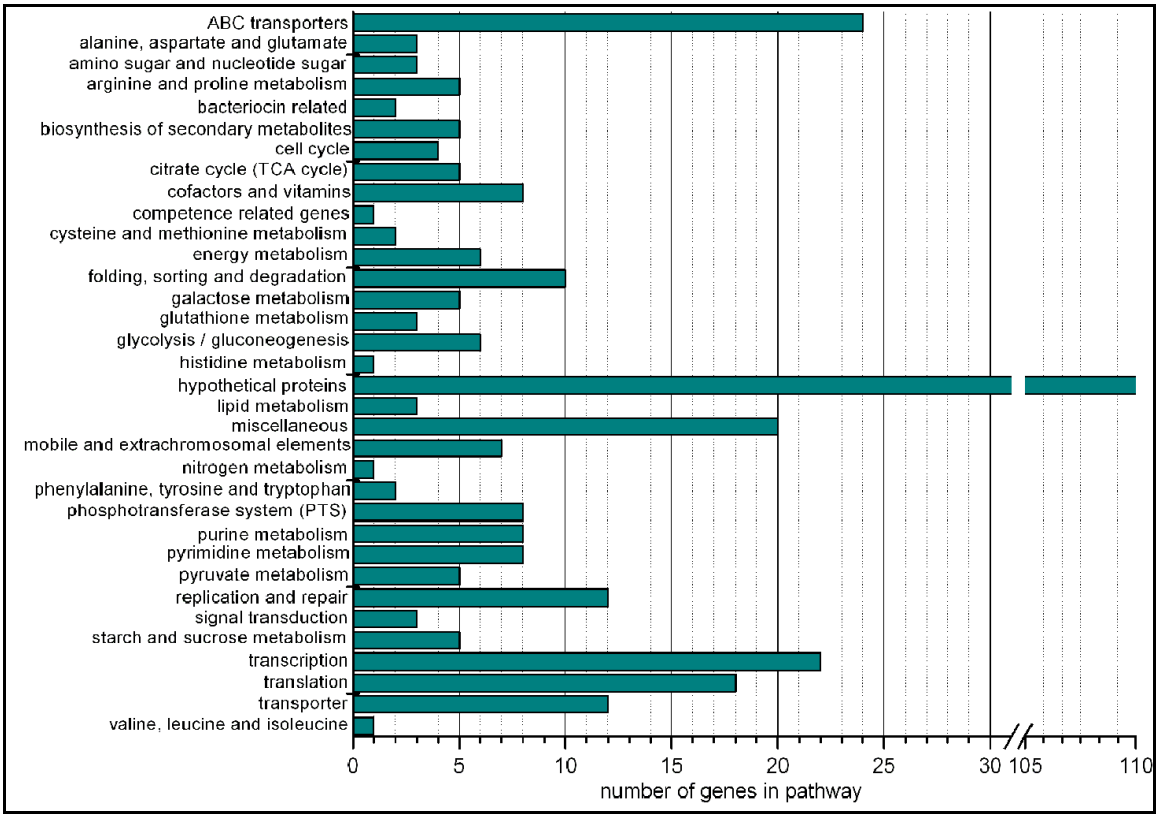

Figure 29. Assignment of differentially expressed genes into metabolic pathways.

Differentially expressed genes of *luxS*$_2$pDLluxS mutant, compared to wildtype, were assigned manually into metabolic pathways according to their annotation. From 340 genes, 193 were down and 147 up-regulated.

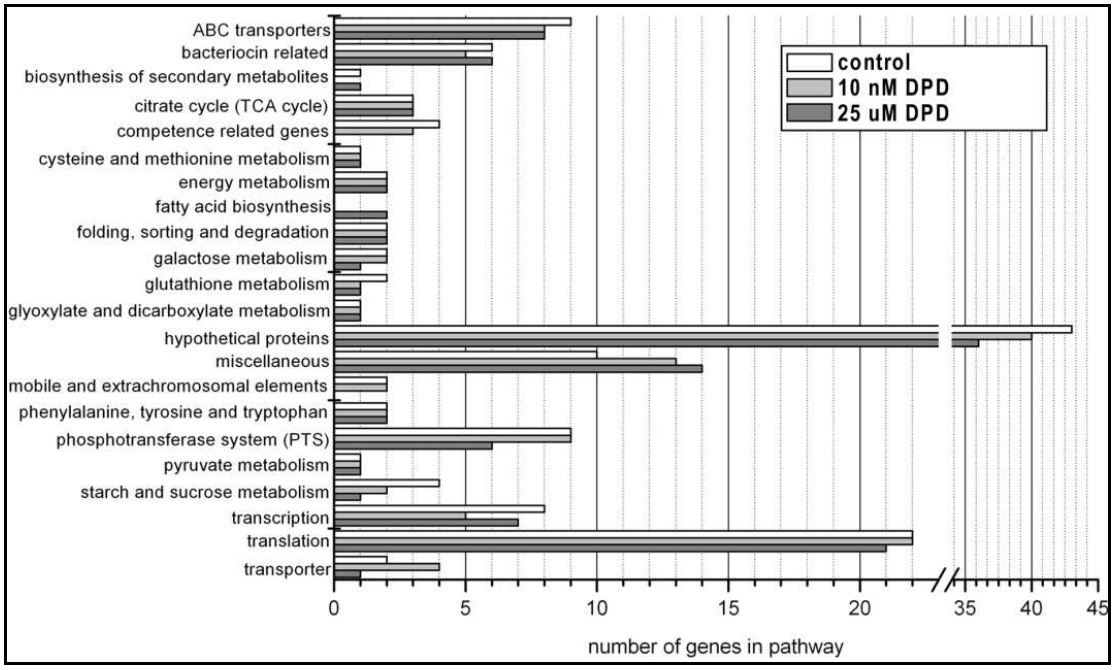

Figure 30. Assignment of differentially expressed genes into metabolic pathways.
Differentially expressed genes of *luxS*$_2$SahH (+ AI-2), compared to wildtype, were assigned manually into metabolic pathways according to their annotation. White bars, *luxS*$_2$SahH - from 136 genes, 49 were down and 87 up-regulated; Light grey bars, *luxS*$_2$SahH + 10 nM AI-2 - from 127 genes, 39 were down and 88 up-regulated; Dark grey bars, *luxS*$_2$SahH + 25 µM AI-2 - from 116 genes, 40 were down and 76 up-regulated.

Influence of the plasmid pDLluxS on *S. mutans*

The investigation of the important virulence traits aciduricity, mutacin production and biofilm formation showed that deletion of *luxS* had not influence but that the presence of pDLluxS caused severe alterations in these traits. An influence of the empty vector could be ruled out since it showed no effect on mutacin production and biofilm formation. Production of AI-2 molecules in the *luxS* deficient *E. coli* DH5α confirmed that the plasmid pDLluxS encodes for a functional LuxS enzyme, therefore we assumed that an increased expression of *luxS* due to a higher copy number might be the reason for the observed effects. However, comparing the expression of the *luxS* gene of strain Δ*luxS*$_2$pDLluxS with the wildtype revealed that its expression was not significantly changed and was comparable to that of the wildtype (table 3). Nevertheless the presence of pDLluxS in Δ*luxS*$_2$ did not restore the expression of the 37 genes that were affected due to the disruption of *luxS* (see table 3 and below). This indicates again the presence of secondary mutations in that strain, albeit they are different from the one occurring in Δ*luxS*$_1$. On the other hand expression of the SAH-hydrolase gene restored the expression of the majority of the 37 changes genes (see below) and thus indicate that these changes were due to disruption of the AMC and do not support the presence of secondary mutations. However, further experimental exploration with different constructs expressing *luxS* are needed for genetic complementation of Δ*luxS*$_2$.

Since the expression data revealed that *luxS* is not over-expressed in Δ*luxS*$_2$pDLluxS suggest the presence of an unknown regulatory element, i.e. a small regulatory RNA (sRNA), in the 500 bp upstream of the *luxS* coding sequence which was cloned in pDLluxS, too. The presence of such sRNA adjacent to *luxS* has also been reported for *E. coli* and *Salmonella typhimurium* (Argaman et al. 2001, Kint et al. 2010). An increased expression of this putative sRNA due to a higher copy number might be responsible for the severe phenotypic defects and altered gene expression, that we observed in the presence of pDLluxS but to confirm this needs also further experimental investigation.

Table 3. Selection of differentially expressed genes in biofilms of the Δ*luxS*$_2$ mutant and its complementation constructs.

Locus tag	Description	Gene symbol	FC luxS$_2$	FC pDLluxS	FC SahH	FC SahH 10 nM	FC SahH 25 µM
***luxS* and heterologous expressed genes**							
SMU.474	S-ribosylhomocysteinase	luxS	**-83**	2,44	**-140**	**-149**	**-64**
sahH	S-adenosyl-L-homocysteine hydrolase	sahH	-1,03	-1,13	**376**	**326**	**219**
aad9	spectinomycin	aad9	1,26	**380**	**798**	**576**	**516**
Glutamate/ornithine metabolism							
SMU.366	glutamate synthase subunit beta	gltD	**1,74**	**2,99**	-1,29	-1,21	-1,15
SMU.663	N-acetyl-gamma-glutamyl-phosphate reductase	argC	**1,88**	**1,92**	1,15	1,28	1,05
SMU.665	acetylglutamate kinase	argB	**2,00**	**2,38**	1,38	1,48	1,35
SMU.664	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	argJ	**1,74**	**1,74**	1,28	1,09	1,12
SMU.666	acetylornithine aminotransferase	argD	**1,75**	**2,44**	1,32	1,56	1,18
SMU.670	aconitate hydratase	citB	**2,03**	**1,84**	**-3,23**	**-2,93**	**-2,76**
SMU.671	citrate synthase	citZ	**2,94**	**2,88**	**-3,21**	**-3,28**	**-3,16**
SMU.672	isocitrate dehydrogenase	citC	**3,00**	**2,83**	**-2,96**	**-3,06**	**-2,92**
SMU.1421	Branched chain alpha-keto acid dehydrogenase subunit E2	pdhC	**1,86**	**2,09**	1,55	1,53	1,19
SMU.1422	Putative pyruvate dehydrogenase E1 component beta subunit	pdhB	**2,12**	**2,32**	1,94	1,78	1,54
SMU.1424	Putative dihydrolipoamide dehydrogenase	pdhD	**1,78**	1,58	1,26	1,3	1,48
SMU.1657c	putative nitrogen regulatory protein PII	-	**2,33**	**3,3**	**-1,87**	**-1,7**	-1,54
SMU.1658	putative ammonium transporter, NrgA protein	nrgA	**2,15**	**2,72**	**-2,17**	**-1,92**	-1,66
Sulfur metabolism							
SMU.930c	LysR regulator	homR	**-1,70**	**-2,48**	1,00	1,17	1,13
SMU.933	putative amino acid ABC transporter,	-	**-2,66**	**-2,71**			

	periplasmic amino acid-binding protein				1,33	1,62	1,67
SMU.934	putative amino acid ABC transporter, permease protein	-	**-2,68**	**-2,02**	1,4	1,68	**1,77**
SMU.935	putative amino acid ABC transporter, permease protein	-	**-2,69**	**-1,88**	1,4	1,68	**1,73**
SMU.936	putative amino acid ABC transporter, ATP-binding protein	-	**-2,64**	-1,62	1,5	**1,75**	**1,87**
Biofilm related genes							
SMU.910	Glucosyltransferase-S	gtfD	1,06	1,08	**1,88**	**1,76**	**1,78**
SMU.1434c	Putative glucosyltransferase		-1,14	-1,34	**1,70**	**1,72**	1,68
SMU.22	Glucan binding protein b	gbpB	-1,13	-1,46	**1,75**	**1,81**	**1,73**
SMU.1004	Glucosyltransferase-I	gtfB	-1,52	**-2,92**	1,35	1,41	1,65
SMU.1005	Glucosyltransferase-SI	gtfC	-1,55	**1,78**	1,3	1,41	1,34
SMU.1396	Glucan binding protein C	gbpC	-1,43	**-1,83**	-1,67	1,53	-1,43
SMU.610	Cell surface antigen SpaP	spaP	-1,42	**-2,62**	-1,19	-1,14	-1,12
SMU.410	Putative transcriptional regulator	brpA	-1,22	**-1,78**	-1,15	-1,07	-1,10
Bacteriocin related genes							
SMU.150	Hypothetical protein	nlmA	1,0	1,04	1,5	1,66	1,56
SMU.151	Hypothetical protein	nlmB	1,02	1,17	**1,9**	**2,08**	**1,87**
SMU.423	Hypothetical protein		1,03	1,08	1,42	1,68	1,67
SMU.1895c	Hypothetical protein		-1,01	1,49	**2,2**	**2,06**	**2,13**
SMU.1896c	Hypothetical protein		-1,04	1,4	**2,07**	**1,87**	**2,04**
SMU.1902c	Hypothetical protein		1,23	1,22	**2,14**	**2,78**	**2,43**
SMU.1905c	Hypothetical protein		1,16	1,22	**1,81**	**1,84**	**1,89**
SMU.1913	Putative immunity protein		-1,02	1,0	**1,74**	**1,87**	**1,85**

Genes that were significantly changed (fold change ±1.7, P-value < 0.05) are in bold. See supplementary tables 1-3 for a complete list of genes.

Glutamate metabolism

Comparing the transcriptome of the *luxS*$_2$ mutant with its parental strain revealed that many of the differentially expressed genes were related to the glutamate/ornithine metabolism.
First of all, the pathway converting oxaloacetate to α-ketoglutarate, involving the pyruvate dehydrogenase (*pdh*) and the enzymes encoded in the *citBZC* operon, was up-regulated. Glutamate is formed from α-ketoglutarate and ammonium by the glutamate synthase (*gltD*), which was induced 1.74 fold. Since ammonium is required for this reaction, the up-regulation of the nitrogen regulatory protein PII and the adjacent ammonium transporter (SMU.1657-1658), is in agreement with the increased production of the glutamate synthesis genes. Furthermore, the enzymes of the *arg* operon, involved in the conversion of glutamate to ornithine, were up-regulated, too. Introduction of the SAH-hydrolase gene showed a strong effect on the expression of these genes, restoring them to or even below wildtype levels,

respectively. However, this demonstrates that the changes in gene expression were due to the metabolic role of LuxS and not due to the production of AI-2.
In EHEC *E. coli* it was shown that deletion of *luxS* caused a decreased aspartate production, resulting in a decreased transcription of the *LEE* type III secretion system, which could be restored by the addition of aspartate (Walters et al. 2006). Since aspartate is synthesized by a simple transamination reaction from glutamate, these data also suggest a general involvement of LuxS in the glutamate/aspartate production. As described above, albeit expression of a functional *luxS* gene, strain Δ*luxS*$_2$pDLluxS did not restore the changes in the glutamate metabolism.

Sulfur metabolism

The activated methyl cycle and pathways coupled to it are part of the sulfur metabolism, responsible for the uptake and production of sulfur containing molecules or its precursors. We observed a known regulatory cascade to be down-regulated in the *luxS*$_2$ mutant. The LysR type transcriptional regulator HomR (SMU.930c) was 1.7 fold down regulated in Δ*luxS*$_2$. HomR was shown to positively regulate the expression of the *tcyDEFGH* operon (SMU.932-936), encoding an ABC transporter responsible for the uptake of L-cystine (Sperandio et al. 2010), whose expression was strongly decreased in the absence of *luxS* (table 3). Introduction of the SAH-hydrolase restored or increased the transcription level of these genes to or beyond the wildtype level, respectively, showing that this defect was caused by the absence of LuxS and not AI-2. Again, the genetically complemented *luxS*$_2$ mutant strain failed to restore transcription of these genes to wildtype levels.

Genes related to biofilm formation

The *luxS*$_2$ mutant strain carrying pDLluxS displayed a reduced capacity to form biofilms compared to the wildtype, since it produced a thinner biofilm and the maturation of microcolonies was severely disturbed. This is in agreement with the observed down regulation of the glycosyltransferases, *gtfBC*, which are responsible for the production of water insoluble glucans (Banas and Vickerman 2003). Along with these structural determinants, the glucan binding protein C (*gbpC*), the cell wall-associated adhesin P1 (*spaP*) and the biofilm regulatory protein A (*brpA*) were also down regulated (table 3).
Strain Δ*luxS*$_2$SahH exhibited a different biofilm structure compared to the wildtype. This could be confirmed by the array data showing that the glucosyltransferase (GTF) *gtfD*, responsible for the production of water-soluble dextran (Banas and Vickerman 2003), was up-regulated. Moreover, a putative glucosyltransferase (SMU.1434c) and the glucan binding protein B (*gbpB*) were higher expressed than in the wildtype (table 3), showing that

manipulation of a central metabolic circuit may have strong influence on other (metabolic) pathways.

Genes related to bacteriocin production

According to the mutacin overlay assays a reduction in mutacin production in strain Δ*luxS*$_2$pDLluxS was expected but no reduction in expression of genes for mutacin IV and V was observed in the microarray analysis. However, comparison of both approaches is difficult since the optical density in the overlay assay is very high and that the cells were obtained from an overnight culture, both conditions known to strongly induce bacteriocin production (Okinaga et al. 2010a), whereas the biofilm was inoculated with a low density. Assuming that the 8 hours incubation during biofilm formation was not sufficient to trigger bacteriocin expression in the wildtype explains that mutacin expression in Δ*luxS*$_2$pDLluxS was not decreased compared to the wildtype, as it was seen with the mutacin overlay assay. Confirming the observation that Δ*luxS*$_2$SahH possesses more dead/damaged cells in the early stage of biofilm formation, transcriptome analysis revealed that several (putative) mutacins were up-regulated compared to the wildtype. In addition, the putative immunity protein SMU.1913c was up-regulated in Δ*luxS*$_2$SahH. The *nlmB* peptide of mutacin IV was significantly induced, whereas *nlmA* did not fulfill the cut off criteria. However, reduction of the fold change stringency but retaining the P-value showed that *nlmA* had the same trend of up-regulation as *nlmB*. Induction of the potent mutacin IV and the putative bacteriocins SMU.1895c, SMU.1896c, SMU.1902c, SMU.1905c and SMU.423 (fold change ~1.6, P-value < 0.05) might explain the higher susceptibility for propidium iodide staining within the first hours of biofilm maturation in this construct.

3.4 **Discussion**

The dual role of the LuxS enzyme, being part of a central metabolic cycle and producing the signalling molecule AI-2, makes it difficult to assign effects of a knock-out mutant to the disruption of the metabolic function or to the lack of AI-2 molecules, respectively. With the availability of pure chemically synthesized AI-2, chemical complementation of *luxS* deletion mutants can be carried out, thereby replacing the addition of spent culture supernatant of the wildtype to the *luxS* mutant. Thus, the complementation problem could be partially solved but nevertheless a *luxS* mutant still lacks a functional AMC and therefore might not represent an optimal background for chemical complementation experiments.

Secondary mutations in *luxS* mutants

Lebeer *et al*. and Doherty *et al*. (Doherty et al. 2006, Lebeer et al. 2007) reported the presence of secondary mutations in *luxS* mutants of *L. rhamnosus* GG and *S. aureus*. Since genetic complementation of *luxS* mutations has not often been carried out (Vendeville et al. 2005), secondary mutations might be more frequent in *luxS* mutants than presently reported. We show here the occurrence of unknown secondary mutations in a *S. mutans* UA159 $luxS_1$ mutant, which has been used previously in a microarray study (Sztajer et al. 2008). In this study, we reported that strain UA159 lacking the *luxS* gene had a severe defect in biofilm formation. This observation was not in agreement with other studies using strain UA159 (Huang et al. 2009, Wen and Burne 2004), but can now be explained with respect to the secondary mutations. Furthermore, complementation with pure chemical synthesized AI-2 was carried out with $\Delta luxS_1$ and revealed that the genes *rpoE* and *mleR* might be regulated by AI-2. However, no relationship between AI-2 and these two genes could subsequently be confirmed (unpublished results). Consequently, the results presented in Sztajer *et al*. have to be interpreted very cautiously (Sztajer et al. 2008). Moreover these findings pinpoint the importance of genetic complementation to ensure that the observed phenotypes arose from the appropriate mutation.

Effect of the *luxS* mutation in *S. mutans* UA159 and its genetic complementation

The biofilm transcriptome analysis revealed (i) a role of LuxS in the regulation of the glutamate metabolism and (ii) a negative influence on the expression of one of the three LysR type transcriptional regulators (HomR), that are involved in the regulation of the AMC and its directly coupled pathways (Sperandio et al. 2007, Sperandio et al. 2010). However, these differences did not affect the growth or the main virulence traits of *S. mutans* UA159 $\Delta luxS_2$ under the tested conditions. Contradictory to our results, Wen and Burne reported that a UA159 *luxS* mutant was more sensitive to acid killing (Wen and Burne 2004). Similar to our results they reported that expression of the important biofilm genes *gtfBC*, *gbpB* and *spaP* and the biofilm formation using polysterene as substratum and sucrose as carbon source was not affected in the *luxS* mutant. However, biofilm formation on hyrdoxylapatite disks was shown to be severely disturbed in the presence of sucrose (Wen and Burne 2004). Since they demonstrated that several main virulence traits of *S. mutans* were affected in their study, a genetic complementation would have been needed to confirm that these phenotypes were due to the lack of *luxS* but it was not carried out. However, experimental conditions are also important and may explain the observed differences to our results. but it also implies that the role of LuxS in *S. mutans* UA159 becomes only important under defined conditions and might differ from one laboratory to another.

In order to genetically complement the *luxS*$_2$ mutant, the *luxS* gene and its 500 bp upstream sequence were cloned into the pDL278 shuttle vector and transformed into Δ*luxS*$_2$. Since none of the investigated phenotypes were altered in the *luxS* mutant, it was difficult to assess the effect of genetic complementation. However, the microarray data allowed to observe some small changes in gene expression in the *luxS* mutant, and thus it was possible to determine the effect of genetic complementation on the expression of these genes. However, the strain Δ*luxS*$_2$pDLluxS did not restore the above mentioned differences in gene expression of the glutamate and sulfur metabolism, which were expressed just as in the *luxS* mutant. Moreover, strain Δ*luxS*$_2$pDLluxS exhibited severe differences of all virulence traits tested and a pleiotropically changed transcription profile, which confirmed these differences. More precisely, all tested virulence traits were down-regulated.
Since the microarray showed that expression of the *luxS* gene in Δ*luxS*$_2$pDLluxS was restored back to wildtype levels and that this construct produces a functional LuxS enzyme, makes it difficult to explain that no reversal of the glutamate and sulphur metabolism genes was achieved. Another secondary mutation does not seem reasonable because both pathways were restored upon expression of the SAH-hydrolase, showing that these changes were due to metabolic disruption of the AMC and thus can be clearly assigned to be caused by the lack of *luxS*. However, other *luxS* expressing constructs has to be applied to verify this assumption and to achieve a successful genetic complementation of the *luxS* mutation.
Recently Kint *et al*. demonstrated that not deletion of *luxS* was responsible for a defect in biofilm formation in *Salmonella typhimurium* but rather an interference with expression of the small regulatory RNA, MicA, which is located within the intergenic region upstream of *luxS*. A balanced level of MicA was shown to be essential for biofilm maturation in *Salmonella* (Kint et al. 2010). A sequence similar to MicA cannot be found in the upstream region of the *S. mutans luxS* gene. However, assuming the presence of another, unknown regulatory RNA, within the upstream region of *luxS*, that is expressed 20-30 fold higher according to the copy number of pDL278 (Burne et al. 1999) might give an explanation of the strong influence of pDLluxS, whereas the empty vector had no influence on the tested virulence traits. This hypothesis requires experimental verification.

Physiological complementation of the *luxS* mutation by the alternative enzyme SAH-hydrolase

Expression of *sahH* in the *luxS*$_2$ mutant resulted in successful complementation i.e. of the glutamate and sulfur metabolism. In some instances an overcompensation of the *luxS* mutation was observed, i.e. opposite expression of these genes (table 3). Thus, the physiological complementation worked, but a fine tuning of the expression of *sahH* is essential to obtain wildtype levels of transcription. Heterologous expression of the SAH-

hydrolase in a *luxS* deletion mutant has also been performed in *E.coli* and was shown to restore the expression of the LEE type III secretion system, even beyond wildtype levels (Walters et al. 2006) confirming that conclusion. However, we also showed that introduction of the SAH-hydrolase into *S. mutans* caused differences in some of its main virulence traits. Since SahH is an enzyme and has no regulatory properties, the observed effects must be the result of metabolic shifts caused by the strong *sahH* expression. Thus, a system with a more balanced *sahH* expression would be needed for optimal physiological complementation of the disrupted AMC.

Chemical complementation

For complementation with pure chemically synthesized AI-2 we used concentrations that have been described to have an effect on several phenotypes in various Streptococci (Ahmed et al. 2007, Ahmed et al. 2008, Rickard et al. 2006) but did not observe any effects under the conditions tested. However, studies that successfully performed complementation experiments reported that AI-2 affected gene expression or phenotypes only in a very narrow concentration range. Moreover, the amount of signal molecules tested differed over several orders of magnitude between the studies (Rickard et al. 2006, Zhao et al. 2003, Shen et al. 2010). Finding the correct culture conditions, concentration and timing of AI-2 addition make complementation experiments apparently very sophisticated, leaving the door open for future experiments, even in bacteria were complementation failed so far.

Species specific differences

Important to note are the studies from Merritt *et al*. and Yoshida *et al*., which used the *S. mutans* strain GS-5 as a parental strain and observed severe defects in biofilm formation after deletion of the *luxS* gene (Merritt et al. 2003, Yoshida et al. 2005), under conditions that did not affect biofilm formation in the *luxS* mutant of strain UA159. A genetic complementation was not carried out in both studies. Thus, the influence of LuxS on biofilm formation in both strains differs strongly and one should be careful to transfer results obtained with one strain to another.

Conclusions

The aim of this study was to complement a *S. mutans* UA159 *luxS* mutant in different ways to separate the metabolic influence of the LuxS gene from the role of the signalling molecule AI-2. We identified secondary mutations which were responsible for the observed phenotypes in a previously published $luxS_1$ mutant (Sztajer et al. 2008). Construction of a new *luxS* strain and re-analysis of its phenotype revealed that LuxS had no big influence on the main

virulence traits of *S. mutans* UA159 but affected its glutamate and sulfur metabolism in a subtle way not reflected in growth. The construct used for genetic complementation failed to complement these changes in gene expression. Moreover, it showed severely impaired virulence phenotypes, since *luxS* expression was restored to wildtype levels raises the question if an unknown regulatory element in the upstream region of *luxS* might have been responsible for these effects. Heterologous expression of *sahH* in the *luxS*$_2$ mutant restored the expression of genes that were changed in Δ*luxS*$_2$ but beyond wildtype levels. Moreover, a huge set of genes were differentially expressed compared to the wildtype or *luxS*$_2$ mutant. This presumably resulted in the observed phenotypic differences in acid tolerance and biofilm formation compared to the wildtype. The addition of various concentrations of pure AI-2 to Δ*luxS*$_2$SahH had no influence on gene expression or on the phenotypes tested. In summary, the data show that AI-2 did not play a role as a signaling molecule in *S. mutans* under the conditions used in this study.

3.5 **Outlook**

Introduction of the plasmid pDLluxS harbouring the coding sequence of *luxS* and 500 bp of its upstream region did not complement for the effects of the *luxS* mutation observed in gene expression. Thus, at least two additionally plasmids has to be constructed to achieve the genetic complementation, (i) increasing the portion of the upstream region to ensure that all regulatory elements needed for *luxS* expression are involved, (ii) expression of *luxS* from a constitutive promoter. In order to confirm the presence of a sRNA in the upstream region of *luxS*, this fragment has to be cloned into several plasmids with different copy numbers to verify (i) the presence of a putative sRNA and (ii) to see its influence with increasing copy number or expression, respectively. The presence of a sRNA has be confirmed i.e. by northern blot and its exact position has to be mapped by primer extension and RACE PCR. If the occurrence of the sRNA can be confirmed it will be to our knowledge the first sRNA described in *S. mutans*. Overexpression and silencing of the sRNA, by introduction of an anti-sense RNA, and a global analysis has to be carried out to disclose the exact role of this putative sRNA in *S. mutans*.

CHAPTER IV

Subpopulation specific transcriptome analysis of CSP induced *Streptococcus mutans*

André Lemme, Lothar Gröbe, Micheal Reck, Jürgen Tomasch, Irene Wagner-Döbler

Helmholtz-Centre for Infection Research, Research Group Microbial Communication, Inhoffenstr. 7, D-38124 Braunschweig

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4.1 Abstract

CSP mediated competence development in *Streptococcus mutans* is a transient and biphasic process, since only a subpopulation induces expression of ComX in the presence of CSP and activation of the DNA uptake machinery in this fraction shuts down ~3-4 hours post induction. Here we combine, to our knowledge, for the first time in bacteria flow cytometric sorting of cells and subpopulation specific transcriptome analysis of both the competent and non-competent fraction of CSP treated *S. mutans* cells. Sorting was guided by a ComX-GFP reporter and the transcriptome analysis demonstrated the successful combination of both methods because a strong enrichment of transcripts for *comX* and its downstream genes was achieved. Three two component systems were expressed in the competent fraction, among them ComDE. Moreover, the recently identified regulator system ComR/S was expressed exclusively in the competent fraction. By contrast, expression of bacteriocin related genes was at the same level in all cells. GFP reporter strains for ComE and CipB (mutacin V) confirmed this expression pattern on the single cell level. Fluorescence microscopy revealed that some ComX expressing cells committed autolysis in an early stage of competence initiation. In viable ComX expressing cells uptake of DNA could be shown on the single cell level.
This study demonstrates that all cells in the population respond to CSP through activation of bacteriocin related genes. Some of these cells start to activate ComX expression but then again segregate into two subpopulations, one becoming competent and another one that lyses, resulting in intra population diversity

4.2 Introduction

Competence development is a complex process involving complex regulatory networks that trigger the capacity to take up exogenous DNA from the environment (Claverys et al. 2007, Claverys et al. 2009, Dubnau 1991, Johnsborg and Havarstein 2009). This phenomenon occurs in gram negative and gram positive bacteria and is frequently encountered in bacteria of the oral cavity, e.g. *Streptococcus mutans* (Tanzer et al. 2001). *S. mutans* is considered the major etiological agent of dental caries because it is associated with the initiation and progression of dental caries. So far, development of genetic competence is best characterized in *Bacillus subtilis* and the human pathogen *Streptococcus pneumoniae* and differs

significantly between these two species (Claverys et al. 2009, Dubnau 1991, Johnsborg and Havarstein 2009).

At the onset of the stationary phase, cells of *B. subtilis* differentiate into several distinct cell types, e.g. ~10% of the cells initiate competence development. This phenomenon of phenotypic variation of a clonal population has been referred to as "bistability", due to the dual stable pattern of gene expression in genetically identical cells (Dubnau and Losick 2006). According to Ferrell, two mechanisms exist to manifest bistability in a bacterial population (Ferrell, Jr. 2002). One is the principle mechanism of competence initiation in *B. subtilis* and is described below. It requires the presence of a positively auto regulated transcriptional activator, which responds to itself in a non-linear manner. Specifically, above a certain threshold of the regulator a hypersensitive change in gene expression is induced due to the activation of a positive auto feedback loop. Consequently, this results in high levels of the regulator, and its controlled genes will be activated (Avery 2005). Cells that do not exceed the threshold will remain inactive, resulting in segregation of the clonal population. In *B. subtilis*, ComK fulfils these criteria and induces the expression of all genes in the competence pathway in a subpopulation.

Whether a cell exceeds the threshold of the regulator concentration to activate the positive feedback loop is determined by noise, or random fluctuations, in the expression of the *comK* gene. In *B. subtilis*, this can be artificially manipulated by inducing the expression of *comK* or by promoting the stabilization of ComK. Due to the non-linear autoregulatory properties of ComK, bistability will remain but the number of competence initiating cells can be varied in that way (Avery 2005).

So far, there is no evidence for bistability in competence development in *S. pneumoniae*. With laboratory strains, routinely ~100% of competent cell could be obtained (Claverys and Havarstein 2007).

During growth, *S. pneumoniae* releases the competence stimulating peptide CSP (encoded by *comC*), using the ComAB secretion apparatus. Interaction of CSP with the membrane embedded histidine kinase ComD results in activation of the cognate response regulator ComE through phoshporyl transfer. Activation of the ComDE two component system leads to the expression of ~20 early competence genes (Cheng et al. 1997, Havarstein et al. 1996, Pestova et al. 1996, Ween et al. 1999). Coordinated induction of competence in the whole population is achieved by a positive feedback loop, since ComE activates expression of the *comAB* and *comCDE* operons. However, this feedback loop in this case does not lead to bifurcation of the population. Martin *et al*. discovered that transcriptional read through of the $tRNA^{Arg5}$ gene upstream of *comCDE* was crucial to maintain a sufficient level of ComDE, ensuring that all cells can initiate competence when the threshold of CSP is exceeded. Inactivation of this transcriptional read through resulted in segregation of the culture and only 25% started expression of late competence genes (Martin et al. 2010). Among the early competence genes is the alternate sigma factor ComX. ComX is required for the induction of

the late competence genes, which includes e.g. the genes necessary for DNA uptake and processing.

S. pneumoniae contains a paralogue of the ComABCDE system, designated BlpABCRH. BlpRH constitutes a two component system which is activated by the auto-inducing peptide BlpC (similar to CSP and ComDE). BlpAB was shown to be necessary for the transport of BlpC. The BlpR regulon was shown to comprise several bacteriocin like peptides (de Saizieu et al. 2000, Reichmann and Hakenbeck 2000).

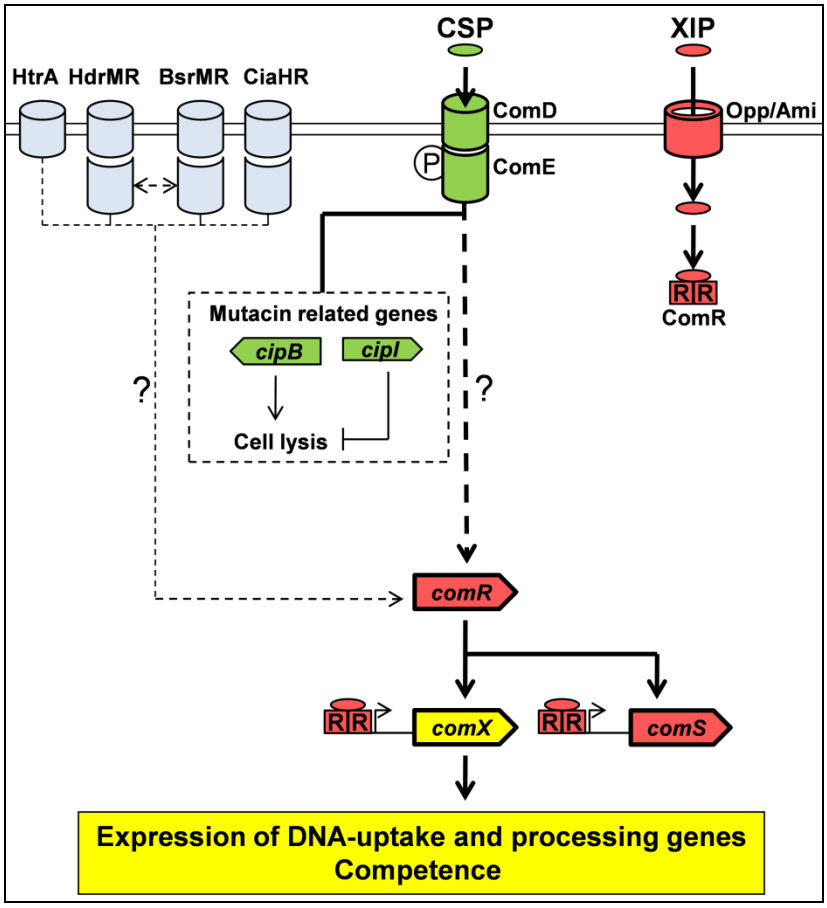

Figure 31. Current model of competence development in *S. mutans*.

Five different systems were shown to influence competence development: The serine protease HtrA, the HdrRM and BsrRM regulatory systems and the two component systems CiaHR and ComDE. The *comC* gene product is the precursor of the CSP (Competence Stimulating Peptide) peptide which is transported and processed via the CslAB (ComAB) transporter (not shown). Accumulation of CSP activates the histidine kinase ComD, which activates its response regulator ComE by phosphorylation. Activated ComE regulates the expression of several mutacin related genes, including the self-acting bacteriocin CipB and its cognate immunity protein CipI. The HdrMR and BsrMR regulatory systems were shown to regulate each other. Furthermore, they were shown to be involved in the regulation of bacteriocin regulated genes (not shown). The signals that are sensed by HtrA, CiaHR, HdrMR and BsrMR are unknown. All signals, including CSP, are integrated by the transcriptional regulator ComR through yet unknown mechanisms. ComR activates the expression of *comS*. The gene product of *comS* is the precursor of the XIP (sigma factor X Inducing Peptide) peptide. ComS is transported into the extracellular environment through an unknown transporter and is processed outside of the cell (not shown). The mature XIP peptide is transported back by the peptide transporter Opp/Ami. ComR in conjunction with XIP

activates the expression of ComS, leading to an auto-catalytic positive feedback, and of the alternate sigma factor ComX. ComX further activates gene expression from late competence promoters and thus drives the expression of DNA-uptake and processing genes leading to genetic competence. (Modified after Mashburn-Warren *et al.* 2010, Perry *et al.* 2009 and Xie *et al*. 2011 (Mashburn-Warren et al. 2010, Perry et al. 2009b, Xie et al. 2010)).

The ComCDE and ComAB system (transport of CSP) of *S. mutans* is more related to the Blp system of *S. pneumoniae*, than to ComABCDE, based on genomic organization and blast identity(Martin et al. 2006). Interestingly, in *S. mutans* the ComCDE system combines the action of the two orthologues in *S. pneumoniae*. Activation of ComE, through CSP and its receptor kinase ComD, leads to induction of competence through the alternate sigma factor ComX and at the same time ComE directly induces a set of bacteriocin related genes (Figure 1) (Ahn et al. 2006, Kreth et al. 2005, Kreth et al. 2006, Kreth et al. 2007, Li et al. 2002, van, Jr. 2005).

Interestingly, disruption of *comE* in *S. pneumoniae* completely abolishes transformation, suggesting that ComE is exclusively responsible for ComX activation. Deletion of *comE* in *S. mutans* affects only the CSP inducible competence initiation, a basal level of competence remains (referred as to CSP independent competence), suggesting that the CSP/ComDE system is one of several signalling pathways used to activate ComX (Ahn et al. 2006). Indeed, under conditions of biofilm growth the HdrRM system was shown to contribute to competence development through activation of ComX by a yet unknown signal (Figure 1) (Okinaga et al. 2010a). Moreover, microarray analysis revealed that both regulators, ComE and HdrR, activate a large set of overlapping genes. (Okinaga et al. 2010b, Okinaga et al. 2010a). Recently, Xie *et al*. identified another regulatory system, designated BsrRM, that primarily regulates bacteriocin related genes but also affects the HdrMR system and thus indirectly contributes to competence development (Xie et al. 2010) (Figure 1).

In *S. mutans*, no binding motif for ComE is present in the promoter region of ComX, suggesting a missing link between both regulators and ComX. This gap was filled by a recent study, which identified a new peptide regulator system (ComS/R) that acts downstream of ComE and activates directly ComX (Mashburn-Warren et al. 2010). Deletion of ComR completely abolished competence, suggesting that signals from all regulatory circuits (CSP dependent and independent) are integrated at the level of ComR. ComR activates expression of the peptide precursor ComS. ComS is secreted, processed and internalised through the peptide transporter Opp. The processed peptide, designated XIP (sigX-inducing peptide), modulates the activity of ComR which activates expression of ComS and ComX (Figure 31). Using GFP reporter constructs it was shown that in *S. mutans* after addition of CSP mutacin IV was expressed population wide, but that expression of ComX was restricted to some cells, suggesting a bistable switch downstream of bacteriocin induction (Kreth et al. 2005, Perry et al. 2009b).

The scope of this study was to determine the CSP dependent activation of competence on the single cell level. Therefore we used flow cytometry to separate competent from non-competent cells, guided by a ComX-GFP reporter. RNA of both subpopulations was subjected to microarray analysis to disclose transcriptional changes. To our knowledge, such a combination of flow cytometry and microarray analysis has never been carried out before in bacteria. Data from GFP reporter strains for ComE and CipB (mutacin V) and for DNA uptake on the single cell level indicate another bifurcation within the ComX expressing subpopulation into cells committing autolysis and cells that develop competence. Our data provide a first view on the segregation of a CSP induced clonal population of *S. mutans* into three phenotypically distinct subpopulations and show the level within the competence signalling cascade where it occurs and thus expand our knowledge for competence development in *S. mutans*.

4.3 **Results**

4.3.1 Kinetics of gene expression of key competence genes in response to CSP.

To obtain a population wide overview of gene expression of key competence genes, we performed a time series experiment to determine the response in gene expression to the addition of CSP using quantitative real-time PCR. Table 4 lists the genes that were chosen for analysis. Induction of the ComE direct target genes *cipB/I* (mutacin V and its cognate immunity protein CipI) reached almost maximal levels already 15 minutes after addition of CSP, showing that low levels of ComE at the time of induction are sufficient to achieve the maximal induction in response to CSP. The regulatory genes (*comE, comS, comR*) acting upstream of ComX were already induced 15 minutes after induction and achieved their highest expression two hours post CSP addition. By contrast, induction of the alternate sigma factor ComX occurred with a delay being first induced 30 minutes after CSP addition, confirming the observations of Ahn *et al*. (Ahn et al. 2006). Similar to its upstream regulators expression of *comX* was highest two hours post induction.

Table 4. Fold change of gene expression compared to time zero in response to 0.2 µM CSP determined by quantitative RT-PCR.

gene	15 min	30 min	60 min	120 min	240 min
comE	1.75 ± 0.14	2.91 ± 0.35	8.54 ± 1.28	18.58 ± 2.23	1.98 ± 0.22
comR	1.38 ± 0.12	2.68 ± 0.27	2.24 ± 0.29	2.97 ± 0.48	0.97 ± 0.05
comS	2.17 ± 0.13	20.88 ± 2.71	68.3 ± 12.98	144.08 ± 30.26	8.97 ± 1.17
comX	0.88 ± 0.03	1.99 ± 0.12	5.29 ± 0.58	12.48 ± 1.75	0.15 ± 0.02
cipB	27.49 ± 3.02	33.86 ± 4.74	34.91 ± 5.24	41.02 ± 6.56	94.99 ± 16.15
cipI	8.45 ± 0.59	11.44 ± 1.94	10.83 ± 1.63	15.52 ± 2.48	14.22 ± 1.99

Average gene expression and standard deviation of two biological samples.

4.3.2 Expression of *comE* and *comX* was transiently activated and showed separation into two subpopulations.

To determine the induction pattern of the *comE* and *comX* genes upon CSP addition, we constructed firefly luciferase reporter plasmids for both genes. Figure 32 shows that both genes were expressed transiently, reaching their highest values around 2-2.5 hours post induction, confirming the qPCR data. The delayed induction of ComX compared to ComE cannot be seen because the first sample for luciferase measurement was taken 30 minutes after CSP addition, where both genes were already induced. Using a ComX-GFP fusion, we showed that expression of ComX was restricted to a part of the culture. This phenotypic variation in ComX expression and the averaged GFP intensity of the whole culture is shown in figure 33. Throughout all the experiments we observed that approximately 30-50% of the cells were expressing GFP. In comparison to the luciferase reporter it took approximately 30 minutes longer (1h post induction) until the first cells became detectable in the fluorescence microscope after addition of exogenous CSP. Additional 30 minutes for chromophore maturation were needed to obtain a detectable signal in a fluorescence microtitre plate reader. Since mRNA levels were highest after 2h post induction and a suitable amount of detectable GFP had been produced, we decided to separate both subpopulations using flow cytometry between 2-2.5 hours after addition of 0.2 µM CSP.

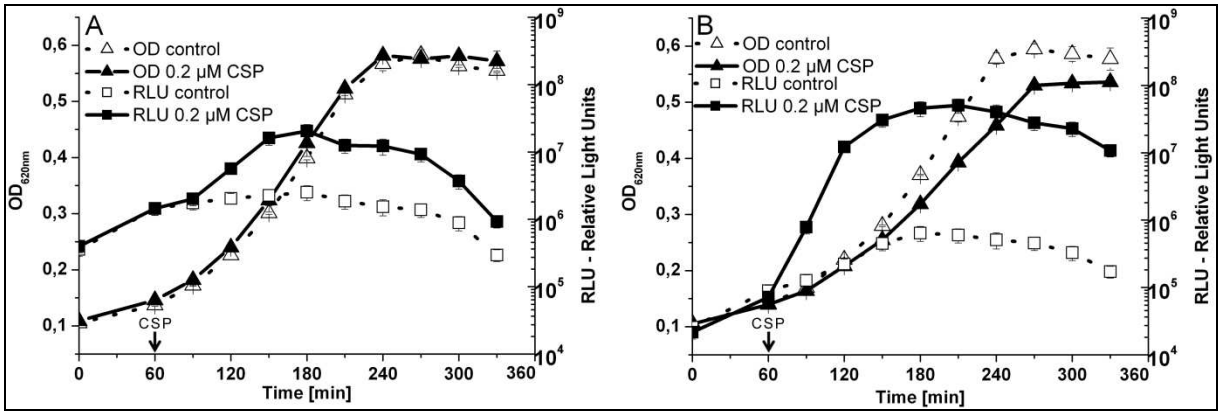

Figure 32. Transient induction of *comE* and *comX* by CSP.

Growth characteristics and luminescence development of the *comE* (A) and *comX* (B) promoter-luciferase fusion in the absence and presence of 0.2 µM CSP. CSP was added after one hour of incubation. Figures show the mean and standard deviation of at least two independent experiments.

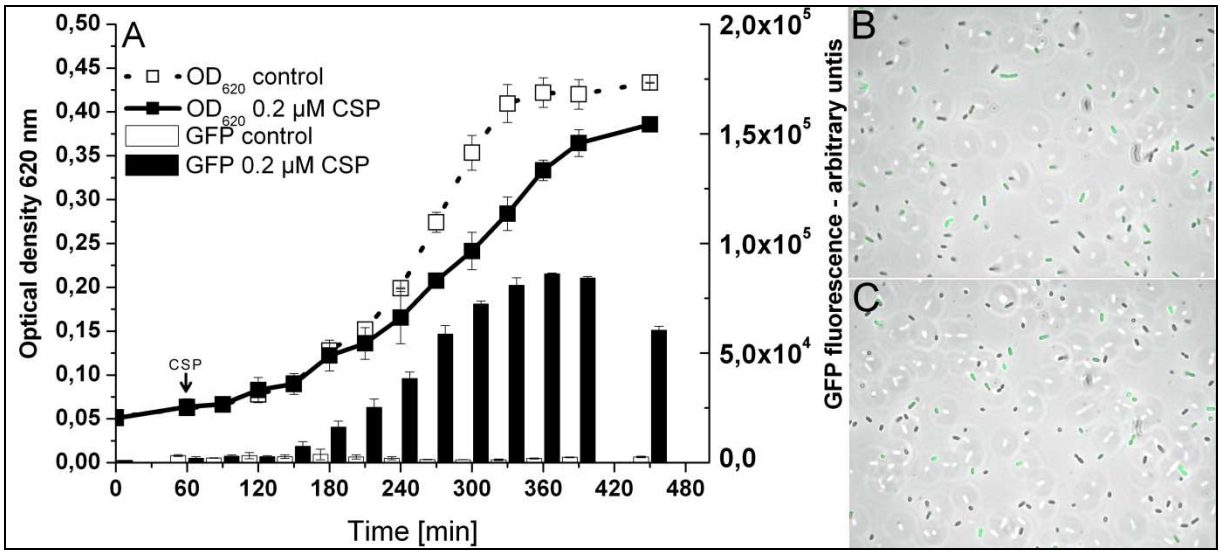

Figure 33. Subpopulation specific induction of SMGFPcomX in the presence of CSP.

Growth curves and development of GFP fluorescence (A). CSP (0.2 µM) was added 1h after inoculation. Figures show the mean and standard deviation of two independent experiments. Fluorescence microscopy of CSP induced (sonicated) cells 2h (B) and 6h (C) post induction.

4.3.3 Both subpopulations could be successfully separated using flow cytometry.

Prior to flow cytometry, the chains of *S. mutans* were disrupted using a sonication treatment. In pre-experiments we tested the ability to disrupt chains by sonication and checked for membrane integrity by staining the cells with propidium iodide (PI, a fluorescence dye staining DNA which does not penetrate intact membranes and is therefore used for detection of damaged/dead cells). No influence of sonication was noticed, e.g. there was no increased number of PI stained cells. Using sonication it was possible to break down the majority of chains to single or doublet cells (Supplementary figure 1).

For calibration of the FACSAria^{TM} II, we first analysed sterile uninoculated media to determine the particle background of the media (Figure 34a). Afterwards, media containing bacteria were used to determine cells/particles of inoculated media and for setting the voltages and gates (Figure 34b). For discrimination of chains or other aggregates FSC-W and SSC-W were enabled and gating was carried out more stringently. Not induced cells reached a GFP fluorescence intensity of approx. 800 units (Figure 34c). To ensure not to collect GFPminus cells with a high auto fluorescence, we set the gate for GFPplus cells at this threshold. The GFPminus population was gated below 300 GFP fluorescence units. The fraction between gate P4 and P5 was not collected.
The histogram in figure 34d represents the GFP intensity of all analysed cells of gate P3. A bi-modular distribution of “dark” and GFP expressing cells became clearly apparent. However, with respect to the amount of cells in both fractions, this pattern was quite dynamic, depending on the fitness of the pre-culture (Supplementary figure 2). Analysis of both separated subpopulation is shown in figure 34d. The majority of “dark” or GFP expressing cells could be successfully separated from each other.

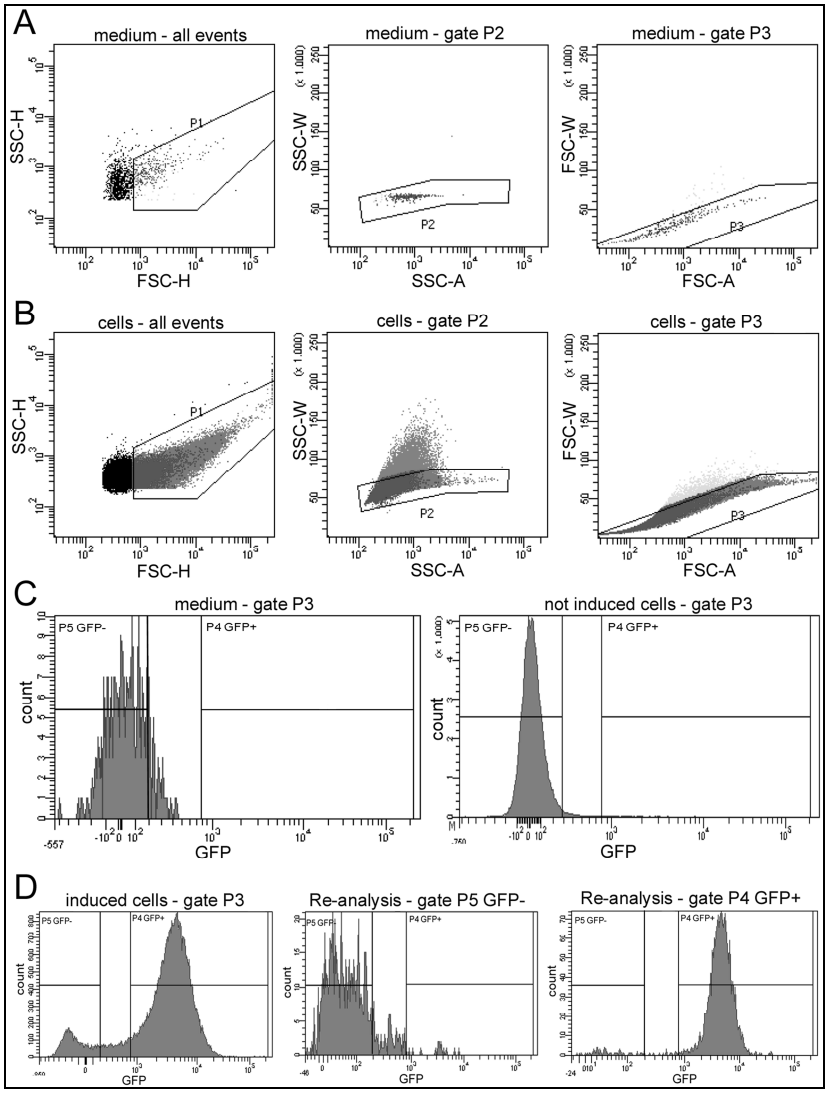

Figure 34. Scatter plots and GFP fluorescence distribution of *S. mutans* cells analysed by flow cytometry.
A: Side- and forward scatter plots of uninoculated media, with the corresponding gates for cell sorting. **B**: Side- and forward scatter plots of inoculated media, with the corresponding gates for cell sorting.
C: GFP intensity from media and not induced cells of the gate P3. Left, intensity of un inoculated media; right, intensity of uninduced SMGFPcomX cells.
D: GFP intensity of induced SMGFPcomX cells of gate P3 and re-analysis after sorting.

4.3.4 Microarray analysis showed an enrichment and depletion pattern of *comX* transcripts in the subpopulations, compared to the mixed population.

The aim of the ComX-GFP guided separation of both subpopulations was to compare their individual gene expression with the transcriptome of induced and non-induced mixed populations, respectively. Below, GFP expressing cells will be designated GFPplus or competent fraction/subpopulation and non GFP expressing cells will be referred to GFPminus or non-competent fractions, respectively.
Sorting resulted in an enrichment (13.8 fold) of *comX* transcription in the GFPplus population, and a depletion (2 fold) in the GFPminus cells compared to the mixed population

(3.6 fold) (table 5 and supplementary table 5). The two fold induction of *comX* in the GFPminus population may reflect a possible carryover of GFPplus cells during the sorting process; alternatively, it might have been caused by naturally competent cells (independent of CSP), whose *comX* transcription level was insufficient for detectable GFP fluorescence. Another possibility would be stress triggered competence induction through the process of sorting, which cannot occur to the mixed populations because they were not subjected to flow cytometry.

Genes that were significantly changed in at least one of the populations are listed in supplementary table 5 and a selection of genes is presented in table 5. Highlights are discussed below. Additionally, a direct comparison of the GFPplus and GFPminus subpopulation was carried out. The direct comparison of both fractions did not reflect the large increase of gene expression in response to CSP in the competent (GFPplus) fraction in a proper way due to the weak enrichment of *comX*, its downstream genes and other known regulators (see below) in the GFPminus subpopulation. Thus, the fold change values are considerably smaller compared to the non CSP-induced control, and smaller changes may be overlooked. For better comparison, the values of all four conditions are listed together. Genes that were significantly changed (fold change +/- 1.8, P-value < 0.05) are marked in bold. The results for some genes were confirmed using quantitative real time PCR and are listed in supplementary table 5. The enrichment or depletion of transcripts in the GFPplus or GFPminus population could be confirmed by qPCR. However, there was a considerable difference in the amount of transcripts in both biological samples yielding a high standard deviation. For are better comparison we inserted the individual results of the qPCR for both biological samples in the supplementary table 5.

Table 5. Selected genes that were significantly changed (log2 fold change > 0.85 and P-value < 0.05) after cytometric sorting.

Locus Tag SMU.#	Description	Gene symbol	Fold Ind. mixed	P-Value Induced mixed	Fold GFP-	P-Value GFP-	Fold GFP+	P-Value GFP+	Fold GFP+/ GFP-	P-value GFP+/ GFP-
Regulatory functions										
61	ComR	*comR*	1.4	1.70E-02	1.1	2.27E-01	**1.8**	**1.31E-03**	1.4	**4.9E-01**
Shp61	ComS within intergenic region 51	*comS*	**15.37**	**1.11E-04**	**6.59**	**5.86E-04**	**81.03**	**1.26E-05**	**11.76**	**7.48E-04**
1916	histidine kinase of the competence regulon, ComD	*comD*	**3.95**	**9.95E-04**	**2.62**	**4.45E-03**	**6.99**	**2.13E-04**	**2.09**	**2.36E-02**
1917	response regulator of the competence regulon, ComE	*comE*	**3.87**	**4.23E-04**	**2.54**	**2.16E-03**	**7.31**	**7.47E-05**	**2.31**	**7.37E-03**
1997	ComX1, transcriptional regulator of competence-specific genes	*comX1*	**3.56**	**2.58E-02**	**2.01**	1.40E-01	**13.79**	**1.50E-03**	**4.96**	**2.48E-02**
Transformasome related										

498	late competence protein	*comF*	**9.37**	**3.86E-04**	**3.60**	**4.25E-03**	**31.81**	**5.38E-05**	**6.93**	**2.28E-03**
499	late competence protein	-	**12.60**	**3.65E-04**	**4.82**	**2.88E-03**	**47.99**	**5.36E-05**	**8.35**	**8.07E-03**
539c	signal peptidase type IV	-	**2.85**	**4.14E-03**	**1.77**	**3.91E-02**	**8.75**	**1.75E-04**	**4.32**	**3.73E-03**
625	competence protein	*comEA*	**20.29**	**2.10E-04**	**7.12**	**1.38E-03**	**70.87**	**4.32E-05**	**8.26**	**4.83E-03**
626	competence protein	-	**18.62**	**1.64E-04**	**6.64**	**1.12E-03**	**55.17**	**3.88E-05**	**6.63**	**5.23E-03**
836	hypothetical protein	-	**9.93**	**7.66E-04**	**3.83**	**7.22E-03**	**34.45**	**1.11E-04**	**6.61**	**3.26E-03**
1979c	hypothetical protein	-	**16.28**	**1.38E-04**	**5.69**	**1.13E-03**	**44.87**	**3.35E-05**	**6.83**	**3.61E-03**
1980c	hypothetical protein	-	**37.48**	**1.92E-05**	**11.83**	**1.11E-04**	**111.95**	**5.66E-06**	**8.30**	**1.50E-03**
1981c	hypothetical protein	-	**41.42**	**1.52E-05**	**13.06**	**8.33E-05**	**134.85**	**4.25E-06**	**8.47**	**7.13E-04**
1982c	hypothetical protein	-	**35.24**	**1.53E-05**	**11.54**	**8.57E-05**	**116.79**	**3.99E-06**	**8.52**	**6.48E-04**
1983	putative competence protein ComYD	*comYD*	**23.54**	**1.14E-04**	**7.52**	**8.42E-04**	**74.40**	**2.75E-05**	**8.08**	**3.90E-03**
1984	putative competence protein ComYC	*comYC*	**40.28**	**1.58E-05**	**12.21**	**9.46E-05**	**140.37**	**4.12E-06**	**9.17**	**5.54E-04**
1985	ABC transporter ComYB	*comYB*	**30.59**	**2.92E-05**	**9.49**	**1.97E-04**	**91.35**	**8.13E-06**	**7.93**	**5.71E-04**
1987	ComYA; late competence gene	*comYA*	**44.78**	**1.51E-05**	**13.28**	**8.81E-05**	**164.36**	**3.86E-06**	**9.63**	**7.10E-04**
Putative Bacteriocins, Immunity Proteins, Bacteriocin Island										
150	hypothetical protein	*nlmA*	**6.53**	**3.84E-04**	**e.65**	**5.49E-04**	**7.29**	**2.98E-04**	1.10	7.16E-01
151	hypothetical protein	*nlmB*	**6.45**	**5.51E-04**	**5.67**	**7.56E-04**	**7.01**	**4.55E-04**	1.04	8.81E-01
423	hypothetical protein	-	**6.66**	**2.34E-04**	**5.92**	**3.11E-04**	**7.25**	**1.92E-04**	1.04	8.58E-01
925	hypothetical protein	*cipI*	**6.18**	**5.92E-05**	**5.20**	**9.33E-05**	**8.75**	**2.66E-05**	1.38	1.10E-01
1892c	hypothetical protein		0.87	6.18E-01	**0.55**	7.56E-02	**0.46**	**3.52E-02**	0.71	1.82E-01
1902c	hypothetical protein	-	**2.83**	**7.64E-04**	**2.80**	**8.09E-04**	**3.13**	**5.09E-04**	0.96	8.32E-01
1903c	hypothetical protein	-	**4.90**	**1.04E-04**	**4.83**	**1.08E-04**	**5.69**	**6.90E-05**	1.00	9.90E-01
1904c	hypothetical protein	-	**4.52**	**3.74E-04**	**4.35**	**4.19E-04**	**5.04**	**2.73E-04**	0.99	9.47E-01
1905c	putative bacteriocin secretion protein	-	**4.83**	**2.74E-04**	**4.75**	**2.86E-04**	**5.40**	**2.01E-04**	0.96	8.53E-01
1906c	hypothetical protein	-	**6.63**	**1.91E-04**	**6.47**	**2.03E-04**	**6.37**	**2.10E-04**	0.84	5.51E-01
1908c	hypothetical protein	-	**6.38**	**1.91E-04**	**6.13**	**2.11E-04**	**6.40**	**1.90E-04**	0.88	5.48E-01
1909c	hypothetical protein	-	**6.23**	**2.40E-04**	**5.93**	**2.72E-04**	**6.03**	**2.61E-04**	0.88	6.32E-01
1910c	hypothetical protein	-	**6.78**	**2.47E-04**	**6.60**	**2.64E-04**	**6.60**	**2.64E-04**	0.87	6.56E-01
1912c	hypothetical protein	-	**6.07**	**1.52E-04**	**5.61**	**1.86E-04**	**6.06**	**1.53E-04**	0.92	6.98E-01
1913c	putative immunity protein	-	**5.69**	**2.78E-04**	**5.49**	**3.06E-04**	**5.86**	**2.58E-04**	0.89	6.10E-01
1914c	hypothetical protein	*cipB*	**8.61**	**1.02E-04**	**8.60**	**1.02E-04**	**8.24**	**1.12E-04**	0.78	4.58E-01

Genes that are significantly changed (log2 fold change > 0.85 and P-value < 0.05) are in bold

4.3.5 Three two component systems showed a similar enrichment/depletion pattern as *comX*.

Our microarray analysis revealed that transcripts for the two component system *comDE* were enriched in the GFPplus fraction, similar to *comX*. Moreover, we observed an enrichment of two additional two component systems, namely HK/RR 4 (SMU.927-928) and HK/RR 9 (SMU.1964-1965). The induction of these three TCS upon CSP addition is in agreement with a population wide transcriptome analysis carried out by Perry *et al*. (Perry et al. 2009b).

Interestingly, a deletion of HK9 resulted in an decreased transformation efficiency, whereas inactivation of HK4 showed no effect (Levesque et al. 2007). However, the new finding that these TCS are induced in the competent cell fraction suggests that they may take part in the competence development, but their exact role needs further experimental exploration. Using qPCR we confirmed the microarray data for *comE* and *comX* (supplementary table 5).

Interestingly, we found several intergenic regions (IGR) that were differentially regulated in both subpopulations independently of their adjacent genes. Table 6 lists all those IGRs that were either differentially expressed in the mixed population, or GFPplus, or GFPminus subpopulation compared to the non-induced control (cut-off criteria fold change > 2, P-value < 0.05), respectively.
Since their neighbouring genes were not affected, these intergenic regions may represent regulatory RNA molecules and a detailed analysis is under way. In addition supplementary table 6 lists all differentially expressed intergenic regions that were changed comparing the CSP induced samples with the non induced control (cut-off criteria fold change > ±1.8, P-value < 0.05).

Table 6. Intergenic regions that were significantly changed after cytometric sorting and whose adjacent genes were not affected.

Genomic Position	IGR between ORFs (SMU.#)		Fold Induced mixed	P-Value Induced mixed	Fold GFP-	P-Value GFP-	Fold GFP+	P-Value GFP+	Fold GFP+ vs. GFP-	P-value GFP+ vs. GFP-
136886_137007	132	133c	1.73	2.73E-01	**3.43**	**4.15E-02**	1.28	6.00E-01	0.24	1.31E-01
179653_179873	181	182	1.69	7.64E-02	**2.68**	**9.38E-03**	1.97	**3.50E-02**	0.77	4.63E-01
367013_367066	390	391c	1.68	1.33E-01	**2.63**	**2.22E-02**	1.97	6.80E-02	0.76	4.52E-01
375868_376028	401c	402	1.55	5.32E-02	1.74	**2.46E-02**	**2.26**	**5.85E-03**	1.25	4.79E-01
435807_435921	466	467	1.59	2.09E-01	**2.53**	**3.67E-02**	**2.06**	7.75E-02	0.84	7.41E-01
596121_596312	636	637c	1.77	1.13E-01	**2.15**	5.11E-02	**2.19**	**4.74E-02**	0.95	8.98E-01
715015_715084	765	766	**2.34**	9.05E-02	**2.99**	**4.37E-02**	1.46	3.92E-01	0.50	2.15E-01
718674_719212	770c	771c	**4.53**	**4.54E-03**	**3.03**	**1.51E-02**	**17.74**	**2.85E-04**	**4.85**	**3.61E-02**
745624_745833	799c	800	1.46	1.28E-01	**2.14**	**1.52E-02**	1.37	1.85E-01	0.69	2.24E-01
755296_755491	807	809	**2.42**	1.11E-01	**3.18**	5.39E-02	**3.61**	**3.90E-02**	1.11	8.80E-01
759342_759398	813	814	1.67	5.56E-02	**2.08**	**1.68E-02**	1.92	**2.52E-02**	0.95	8.74E-01
837975_838127	883	885	**3.64**	**3.47E-02**	**3.71**	**3.29E-02**	1.86	2.20E-01	0.52	2.20E-01
844419_844621	889	890	**2.06**	**4.32E-02**	**2.50**	**1.95E-02**	1.60	1.37E-01	0.66	2.64E-01
845165_845295	890	891	1.67	5.46E-02	**2.01**	**1.96E-02**	1.48	1.13E-01	0.76	3.30E-01
878575_878770	923	924	1.94	**2.86E-02**	**2.59**	**7.54E-03**	**2.08**	**2.00E-02**	0.82	5.52E-01
896354_896469	944	946	1.66	1.18E-01	**2.07**	**4.36E-02**	1.43	2.40E-01	0.74	4.15E-01
938355_938638	991	992	**2.15**	1.06E-01	**4.15**	**1.54E-02**	**2.61**	5.73E-02	0.68	4.76E-01
996212_996313	1048	1050	**2.13**	6.85E-02	**2.95**	**2.21E-02**	1.91	1.05E-01	0.71	4.59E-01
998766_999060	1052	1053	1.51	2.21E-01	**2.25**	**4.24E-02**	1.69	1.37E-01	0.76	5.59E-01
1008295_1008566	1063	1064c	**2.37**	**4.82E-02**	**3.70**	**1.14E-02**	**3.90**	**9.84E-03**	1.05	9.05E-01
1019708_1019801	1076	1077	**2.03**	5.05E-02	**2.05**	**4.91E-02**	1.56	1.68E-01	0.76	4.76E-01

1021518_1021647	1077	1078c	1.99	**3.50E-02**	**2.29**	**1.88E-02**	**2.00**	**3.47E-02**	0.86	6.68E-01
1138619_1138670	1195	1196c	**2.31**	7.34E-02	**3.46**	**2.12E-02**	**3.40**	**2.22E-02**	1.03	9.59E-01
1139685_1139763	R0092	1197	**2.50**	**1.02E-02**	**2.73**	**7.10E-03**	**2.73**	**7.20E-03**	1.06	8.36E-01
1307188_1307342	1378	1379	**2.07**	6.15E-02	**2.20**	**4.77E-02**	1.61	1.74E-01	0.77	4.93E-01
1381695_1381764	1450	1451	1.76	1.39E-01	**2.57**	**3.34E-02**	**2.95**	**2.10E-02**	1.00	9.95E-01
1759753_1759939	1865	1867c	**2.13**	6.08E-02	**3.12**	**1.57E-02**	1.82	1.13E-01	0.68	3.41E-01
1889423_1889587	2016	2017	1.29	3.09E-01	**2.04**	2.78E-02	1.66	7.95E-02	0.79	4.01E-01
1892786_1893019	2025	2026c	1.51	5.55E-02	1.40	9.90E-02	**2.14**	**6.56E-03**	1.96	**1.59E-02**
1906075_1906218	2035	2036	1.74	1.98E-01	**2.76**	**4.33E-02**	**2.20**	8.96E-02	0.78	5.86E-01

IGR that are significantly changed (fold change > 2.0 and P-value < 0.05) are in bold

4.3.6 Induction of ComS and ComR was restricted to the competent fraction.

In accordance with the recent finding that XIP(ComS)/ComR is the proximal regulator for *comX* induction, both genes showed the same pattern of enrichment and depletion in the two subpopulations (table 5) (Mashburn-Warren et al. 2010). Transcription of ComR has been shown to be 2-3 fold increased due to the presence of CSP, loss of *hdrM*, or overexpression of *hdrR* (Okinaga et al. 2010b, Perry et al. 2009b), confirming the weak induction we observed in our transcriptome analysis. Since *comS* is a newly annotated open reading frame it was not directly included in our array design, but the intergenic region harbouring *comS* was 81 fold induced in the GFPplus fraction and only 6.6 fold in the GFPminus fraction.

4.3.7 Transformasome genes were highly enriched in the competent subpopulation.

The alternative sigma factor ComX is responsible for transcriptional activation of DNA uptake and processing genes, the so-called transformasome (Claverys et al. 2009). As a consequence of *comX* enrichment in the GFPplus subpopulation, the gene transcripts responsible for building and assembling the transformasome were highly enriched (up to 164 fold) in the GFPplus fraction, compared to the non induced mixed population. Due to the weak enrichment of *comX* transcripts in the GFPminus fraction, we observed a weak induction of ComX downstream genes in this fraction, too. However, by comparing the ratio of both separated subpopulations, we obtained a transcriptional enrichment of up to ~10 fold for the ComX downstream target genes, demonstrating the success of the applied approaches. Table 5 lists the genes for DNA uptake and processing based on their orthologoues in S. *pneumoniae*. Recently Okinaga *et al*., identified a gene (SMU.836), which is not part of the transformation machinery of *S. pneumoniae*, but was shown to be essential for DNA uptake in *S. mutans* (Okinaga et al. 2010b). This gene was also enriched in the competent population. Beside other DNA processing proteins, the five genes that are essential for the processing of transformed DNA in *S. pneumoniae* (CoiA, DprA, RecA, SsbB and RadA) followed the same

pattern of enrichment and depletion. (*radA* [SMU.327] was only slightly induced [1.7 fold, GFPplus] and thus did not fulfil the criteria of the microarray data analysis).

4.3.8 Bacteriocin related genes were similarly transcribed in both subpopulations.

In *S. mutans*, bacteriocin production is connected with competence development. The (putative) non-lantibiotic bacteriocins SMU.423, SMU 1906c, mutacin IV (*nlmAB*), CipB (mutacin V) and its cognate immunity protein (CipI) (Perry et al. 2009b), were shown to be directly regulated by ComE, due to the presence of a ComE binding motif in the promoter regions (Kreth et al. 2007, van, Jr. 2005).
Since *comDE* was enriched in the GFPplus subpopulation, it would have been expected that its direct target genes would have been enriched as well. Table 5 lists the putative bacteriocin related genes (according to the oralgen database, http://www.oralgen.lanl.gov/), that were induced by CSP in our study. Surprisingly, in contrast to the transformasome and DNA processing genes, there was no clear enrichment of bacteriocins in the competent fraction. The ratio of both subpopulations shows that there was only little enrichment for the mutacin V immunity protein CipI (*cipI*; SMU.925). By contrast, the self-acting mutacin CipB (mutacin V), as well as the other bacteriocin related genes were equally induced in both subpopulations. To increase the sensitivity of detection, we used qPCR to determine the transcriptional level and confirmed the data for *nlmA*, *cipB* and *cipI* (supplementary table 5). This finding was surprising to us, since the majority of these genes are directly controlled by ComE because of the presence of a ComE binding motif. Due to the enrichment of *comE* transcripts, one would expect a similar pattern for its bacteriocin target genes.
Addition of CSP reduces the growth rate of *S. mutans*. Perry *et al*. demonstrated that this decrease in growth was due to autolysis caused by the action of CipB. Furthermore, they showed that the majority of cells expressing ComX (visualized with a GFP reporter) were susceptible for propidium iodide staining, presumably due to an increased expression of CipB or to an imbalance of CipB/CipI (Perry et al. 2009b). According to their observations, the results from our transcriptional analysis were unexpected. We did not perform a time series experiment to determine the transcriptional pattern of both genes. But at least for the time point under the condition analysed here, it seems very unlikely that cells undergoing competence development triggered their cell death at the same time.

4.3.9 CSP induced autolysis is correlated with weak ComX expression.

To evaluate the correlation of ComX expression and PI staining in more depth, we counterstained CSP induced cells (SMGFPcomX) with PI and visualized them using fluorescence microscopy.

The overlay image in figure 35 represents a typical experiment. When we first analysed the fluorescence images we did not see a correlation between green and red cells, which confirmed our transcriptome data. However, when we increased the green fluorescence intensity for the whole image, weakly fluorescing green cells became visible. The important observation was, that the majority of PI stained cells was weakly fluorescing green and thus expressed GFP to a small extent. In addition to the data shown in figure 35, we tested four different conditions: addition of 0.2 µM or 2 µM CSP, immediately and one hour after inoculation, respectively. Under all tested conditions, we did observe this correlation of weak GFP and PI fluorescence (data not shown).

Autolysis through CipB is presumably mediated through an overexpression of CipB or to an imbalance of CipB/CipI. If some cells produce CipB to a greater extent, one would expect a bimodal distribution of *cipB* expression when single cells are analysed. Using flow cytometry, we observed an unimodal distribution of CipB expression after CSP addition (Figure 36b). Consequently, autolysis must occur due to a different amount of the cognate immunity protein CipI. This conclusion is supported by our transcriptome data which show an enrichment of CipI in the ComX induced subpopulation but an equal expression of CipB in both subpopulations. This is in full accordance with our microscopic observations, showing that moderate to strong GFP expression did not correlate with cell death, because these cells were protected by CipI. The data suggest the presence of an additional subpopulation among the ComX induced cells. During competence development of the CSP responsive subpopulation, some cells were apparently selected to undergo autolysis. This would explain the small amount of GFP because these cells already started the competence cascade and were expressing ComX/GFP. The possibility that some of the cells expressing GFP only weakly were collected in the GFPminus fraction would represent an additional explanation for the weak induction of the competence genes in this fraction.

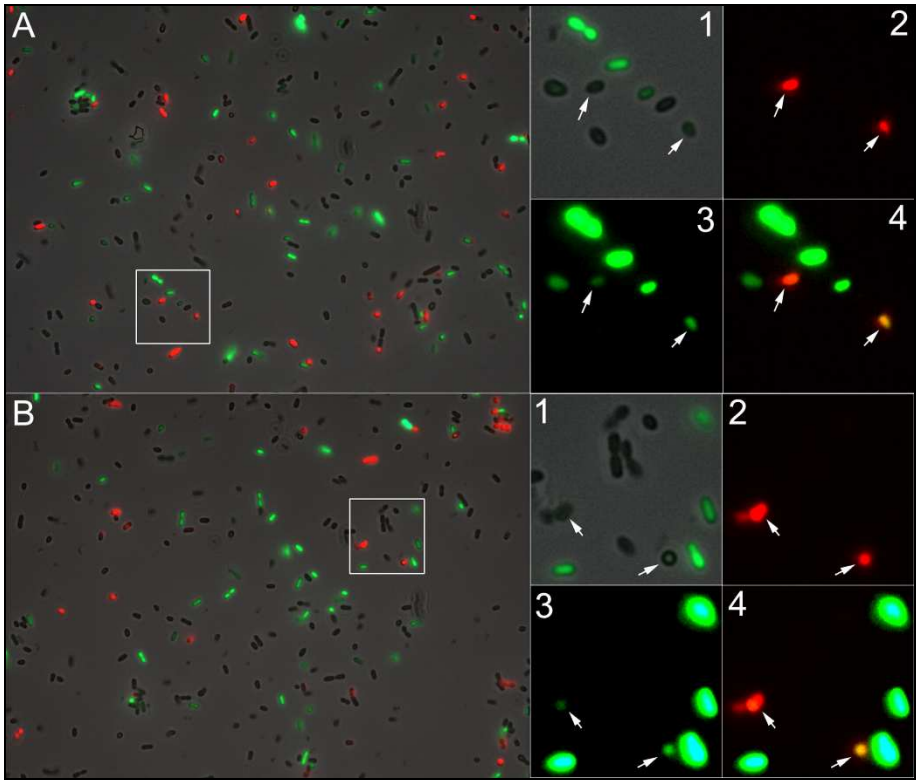

Figure 35. Propidium iodide (PI) counterstaining of CSP induced SMGFPcomX cells.
Cells were 1:10 diluted in fresh media and incubated for one hour before addition of 0.2 µM (A) or 2 µM (B) CSP. Images were recorded two hours post induction. The large images left are overlays of phase contrast, GFP and PI fluorescence. The highlighted area represent cells for every phenotype and is magnified in the right: image 1, overlay of phase contrast and GFP; image 2, PI channel; image 3, increased brightness of the GFP channel; image 4, overlay of bright GFP and PI. White arrows mark cells with a very weak GFP expression that became visible, when the brightness of GFP was increased. Importantly, the majority of dark cells did not show enhanced green fluorescence after brightness modification, confirming that the weak green fluorescence was due to GFP expression and not to auto fluorescence. The majority of red cells showed an enhanced green fluorescence after increasing the brightness.

4.3.10 Fluorescence microscopy confirmed that CipB is expressed in all cells but ComE only in a subpopulation.

Analysis of the subpopulation specific transcription data had indicated that phenotypic variation occurred upstream of ComX, presumably at the step of ComE induction. To confirm these results, we constructed GFP reporter strains for *comE* and *cipB* (mutacin V), which provide a tool to measure protein synthesis rather than gene transcription. Fluorescence microscopy confirmed the transcriptional data, showing that ComE-GFP was only expressed in a subpopulation, whereas CipB-GFP was induced in all cells (Figure 36a). Without addition of CSP, no GFP fluorescence could be observed, showing that the synthesis of CipB and ComE under these conditions was due to CSP addition, not to other stimuli. To confirm that cells expressing ComE-GFP or ComX-GFP were actually competent, we visualized the

uptake of Cy3-labelled DNA. As expected, only cells expressing GFP were able to internalise DNA (yellow cells in Fig 36a), thus confirming their competent state.

4.3.11 Synthesis of GFP from the *cipB* promoter was weak compared to that of *comE* and *comX*.

During microscopy, we observed that cells carrying pALSM04 (P*comX*-GFP) and pALSM34a (P*comE*-GFP) produced more GFP than cells carrying pALSM28 (P*cipB*-GFP). To further confirm this observation, we analysed cells of CSP induced cultures of these strains by flow cytometry.

For measuring the GFP intensity, we set the gate for GFPplus > 400 fluorescence units, to obtain the smallest overlap between low amounts of GFP and high autofluorescence.

As expected, all cells expressing CipB-GFP exhibited higher fluorescence upon induction by CSP and shifted to the right on the x-axis (Figure 36b). By contrast, only a part of the total population of the ComE-GFP and ComX-GFP cells, respectively, showed an increased fluorescence and shifted to the right upon CSP addition. The median intensities for the gated cells (>400 units) were 734 ± 75 for CipB-GFP, 911 ± 33 for ComE-GFP, and 1328 ± 196 for ComX-GFP, confirming the microscopic observation that ComE-GFP and ComX-GFP produced higher amounts of GFP than CipB-GFP and that these genes are exclusively induced in a subpopulation.

The finding that relatively little GFP protein was produced from the *cipB-gfp* mRNA was surprising considering the relative amounts of transcripts for each gene. Figure 36c, shows the relative cDNA abundance, determined by qPCR for both biological replicates of non induced cells and the two induced subpopulations separated by flow cytometry. The relative amount of *cipB* cDNA was ~385 fold higher than that of *gyrA* (in both subpopulations). The level of *comE* in the GFPplus fraction was approximately 18 fold compared to that of *gyrA* and that of *comX* was even lower. Therefore, the strength of the *comE* promoter under these conditions was apparently moderate, and that of *cipB* was very high. It is worth mentioning, that the amount of *cipB* mRNA/cDNA was also very high in the absence of CSP.

Based on the high level of transcription of *cipB*, one would have expected that the *cipB* reporter strain would produce more GFP than the other two.

For each reporter strain, the coding sequence of each gene was replaced by GFP, but promoter sequence and ribosomal binding site remained unchanged (except for an addition of ATC in front of the start codon). Translational efficiency of any mRNA is highly dependent on the nucleotide composition in the translation initiation region (30 bp upstream of the start codon), which determines mRNA conformation and ribosome affinity. Apparently the transcript of *cipB* had a low translational efficiency compared to that of *comE* and *comX* in our reporter construct, but we cannot currently infer the translational strength of the native *cipB* mRNA.

Alternatively, posttranscriptional regulatory events could have taken place, e.g. mediated by small regulatory RNAs.

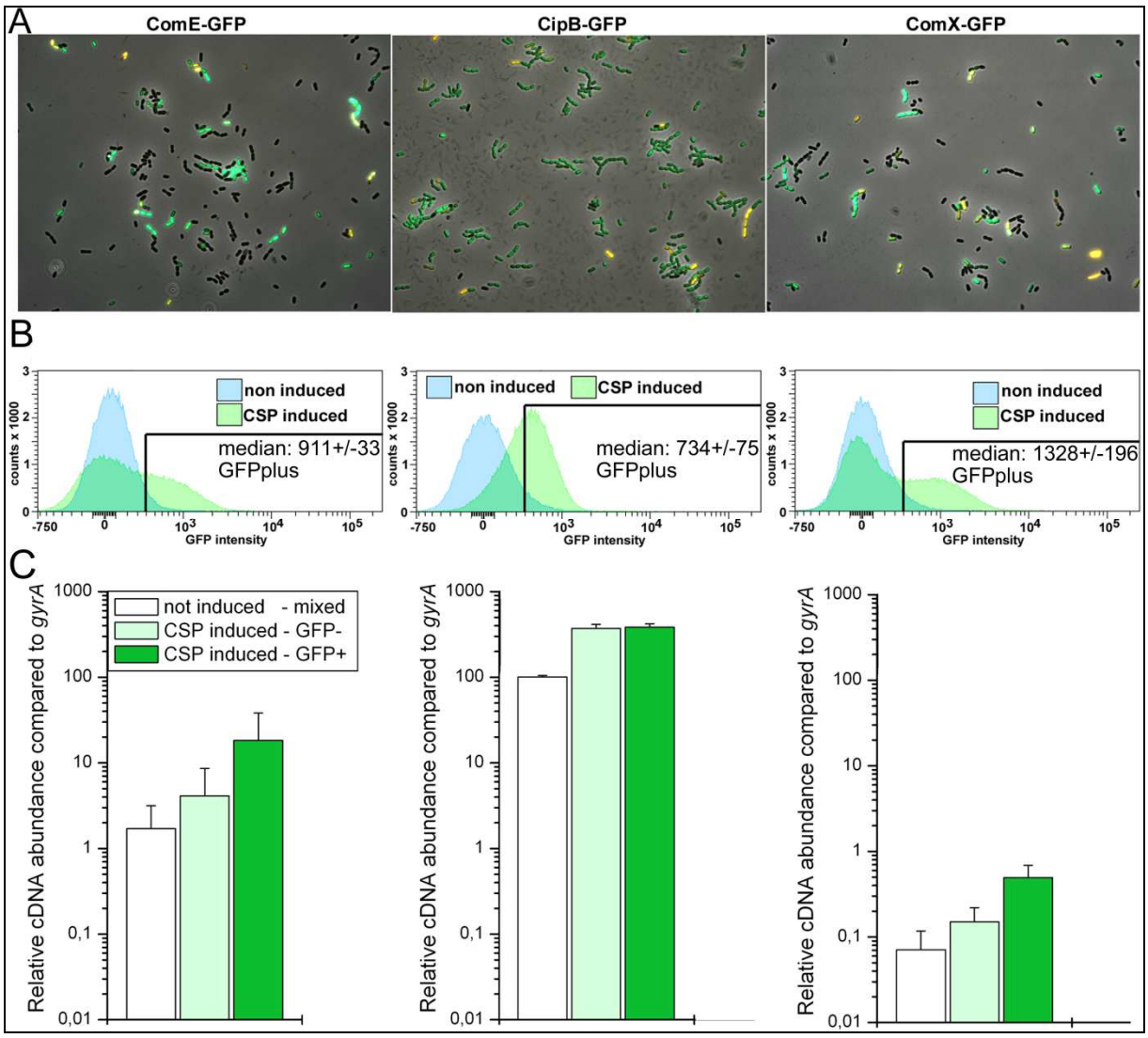

Figure 36. Single cell analysis of *comE*, *cipB* and *comX* expression.

A: Microscopic pictures of CSP induced cells of SMGFPcomE (left), SMGFPcipB (middle) and SMGFPcomX (right). Images of phase contrast, GFP (green, gene expression) and Cy3 (yellow, DNA uptake) fluorescence were merged. **B**: GFP intensity distribution from 100.000 cells of strains SMGFPcomE (left), SMGFPcipB (middle) and SMGFPcomX (right) determined by flow cytometry. The mean GFP intensity and standard deviation of two biological replicates of the GFPplus fraction is shown. **C**: Gene expression analysis with quantitative real time PCR using both subpopulations of FACS separated, CSP induced SMGFPcomX cells and the non-induced mixed population.

4.4 Discussion

Competence development in *S. mutans* is a bistable system and results in intra-population diversity. The analysis of differential gene expression to obtain detailed insights into the different regulation of the subpopulations requires the use of single cell and RNA techniques. Kreth *et al*. and Perry *et al*. for the first time used GFP reporter strains to visualize gene expression on the single cell level in *S. mutans* to study competence development (Kreth et al. 2005, Perry et al. 2009b). However, microarray analysis was averaged over the entire population.

To our knowledge, the combination of flow cytometry and transcriptome analysis of the separated subpopulations has never been done in bacteria. Many efforts have been carried out to improve the existing fluorescence proteins but a remaining problem for coupling reporter gene expression with cellular transcriptome analysis is the delay between synthesis of mRNA, translation and subsequent chromophore maturation. For the GFP used here, it was shown that under control of the arabinose utilizing system at full de-repression the onset of detectable fluorescence was ~ 16 ± 2.5 minutes post induction. However, with decreasing inducer concentration the time until fluorescence became detectable increased up to ~34 ± 10 min (Megerle et al. 2008).

Thus, the time course of CSP mediated competence development in *S. mutans* allowed the combination of both techniques. Competence development in *S. mutans* is a slow process, since highest levels of mRNA for the key regulators *comE* and *comX* are obtained approximately two hours post induction and competence is maintained for another two hours, while competence in *S. pneumoniae* is only observed in a time window of about 40 min after CSP induction (Ween et al. 1999). Thus, there is enough time for GFP maturation in *S. mutans*. We demonstrate here the applicability and the success of combining both approaches to compare the differential gene expression of a competent and non-competent fraction derived from the same population, since mRNA levels for the key regulators ComDE, ComR, ComS ComX and the transformasome genes were highly enriched in the GFPplus fraction. We show that all bacteriocin related genes were similarly expressed in both populations upon the addition of CSP, particularly CipB (mutacin V). Perry and co-workers demonstrated that CipB is a self-acting bacteriocin and that the protein CipI confers immunity against it. Moreover, they showed that the majority of the ComX expressing cells underwent autolysis (Perry et al. 2009b). By contrast, we did not see a correlation of strong ComX expression and autolysis. But under all tested conditions we found that autolysis was indeed correlated with ComX expression but these cells did not produce GFP to a great amount.

Perry and co-workers showed that the immunity protein CipI is additionally regulated by the LiaSR two component system in a cell density dependent manner. Furthermore, they demonstrated that the time of CSP addition to planktonically growing cells was very important, because it influenced the amount of cells that underwent autolysis (Perry et al. 2009a). Different experimental conditions may explain that they observed that nearly all CSP responsive cells committed autolysis. Nevertheless, our data strongly indicate that the CSP

responsive subpopulation itself bifurcates into two subpopulations: one that triggers autolysis and another one that develops the full competence response.
The altruistic behaviour of autolysis would not be favoured by evolution without providing benefits to the remaining bacterial population. One advantage of autolysis is the release of DNA which provides building material for the biofilm matrix and thus increases the ability to form biofilms, which has already been shown for *S.* mutans (Perry et al. 2009a, Petersen et al. 2005). Competence is measured by determining the number of cells that integrate added DNA into the chromosome. In *S. mutans* a transformation frequency of not more than 1-2% can be obtained, regardless of the employed conditions or the source of donor DNA. We show here that the amount of ComX expressing cells in the total population was roughly 30-50%. Our transcriptome data indicate that the DNA uptake machinery in these cells was highly expressed but nevertheless transformation efficiencies in these dimensions could not be achieved. Possibly, internalised DNA was not used for recombination but for repair as it is the case for *S. pneumoniae* (Claverys et al. 2006) or as a source of nutrients. The latter possibility seems unlikely because natural competence in *S. mutans* develops in the exponential growth phase, where nutrients are not exhausted. Another role for the DNA uptake machinery apart from internalising DNA has been demonstrated by Petersen and co-workers (Petersen et al. 2005). They showed that the pilin-like proteins of the DNA uptake protein complex, which are evolutionarily related to type IV pili, are necessary for biofilm formation.
Similar observations were also reported from other bacteria, e.g. *Pseudomonas aeruginosa*, where type IV pili determined the shape of the biofilm architecture (Barken et al. 2008). The transformasome protein ComYB of *S. mutans* is needed for correct assembly of the pilin-like proteins. Deletion of ComYB not only resulted in decreased DNA uptake of 88%, also the formation of the biofilm was severely affected since the remaining components of the multi protein complex could not be assembled correctly (Petersen et al. 2005).
The two component system ComDE represents the major signal transduction system for CSP mediated competence development in *S. mutans*. Deletion of either *comD* or *comE* resulted in no competence induction through the addition of CSP, concluding that activation and induction of ComDE is the first step in CSP mediated competence development (Ahn et al. 2006). The CSP signal is further transmitted to the ComR regulator, which directly activates expression of ComX/SigX (Mashburn-Warren et al. 2010). Our study demonstrates for the first time that bifurcation into competent and non-competent cells occurred before induction of ComX, since the ComE-GFP reporter was expressed only in a subpopulation. It has to remain open which mechanism is responsible for the bifurcation of the population at the level of ComE. It is not known how the transcription of *comE* is regulated, except that it is induced by CSP. The action of ComR can be precluded due to the lack of the highly conserved consensus ComR recognition site in the promoter region of *comE*. Similarly, ComX can be excluded, since there is no binding motif for ComX in the promoter region of *comE* and knock-out of ComX does not affect CSP induced *comE* expression (Perry et al. 2009b).

Adapted from Mashburn-Warren *et al.* (Mashburn-Warren et al. 2010), we present a model that summarizes the current knowledge of CSP mediated competence development (Figure 37) and is described below.

Before the natural accumulation or exogenous addition of CSP the ComDE system is expressed at a basal level but is not activated. Above the threshold of CSP, ComE molecules become phosphorylated and activate expression of the bacteriocin related genes. We assume that due to the presence of the ComE binding box in their promoter regions, a low amount of activated ComE protein is sufficient to drive full expression. Because ComDE is present in all cells at a low concentration, bacteriocins are expressed in the whole population at a high rate. CSP mediated induction of *comDE* transcription, which occurs only in a subpopulation, may require an unknown factor, possibly in combination with population wide variability in the expression of *comE*. Due to the lack of the known ComE binding site in the *comE* promoter region, the presence of a currently unknown factor that is needed for *comE* induction is likely. We suggest that competence initiating cells are selected by the noise in ComE expression and that cells exceeding a certain threshold activate a positive feedback by interaction with an unknown factor. A similar mechanism has been found to control competence in *B. subtilis*, except that ComK directly activates a positive autoregulatory loop (Dubnau and Losick 2006).

We show here that despite a moderate amount of *comE* transcripts a high number of GFP proteins was obtained from the *comE-gfp* promoter fusion. Ozbudak *et al*. demonstrated that a high translational efficiency coupled to a low transcriptional rate resulted in the highest amount of noise (Ozbudak et al. 2002). According to their observations and calculations, large noise in ComE expression would have to be expected supporting the hypothesis that cells might have been selected due to noise in ComE expression.

We suggest that two levels of ComE expression exist in the whole population. Expression below threshold induces bacteriocin synthesis, while above threshold expression triggers induction of *comDE* and *comR* and selects the cells for subsequent activation of ComX. We further suggest that cells that establish a positive feedback for ComE activate (directly or indirectly) ComR, which then induces the expression of *comS* and *comX* and subsequent expression of the late competence genes.

Our data indicate a second bifurcation, inducing autolysis in some cells through the action of CipB. According to the studies of Perry *et al*., the immunity determinant CipI regulated by LiaR was shown to dictate the cell fate of autolysis (Perry et al. 2009a, Perry et al. 2009b), which is in accordance with the enrichment of *cipI* transcripts in the ComX expressing subpopulation. Beside its autolytic properties CipB is a potent mutacin, targeting mainly non streptococcal species (Hale et al. 2005). Thus, CSP mediated competence initiation liberates DNA from competitors in the oral cavity, providing a source either for genetic diversity, or for replenishment of the nucleotide pool that can be used for DNA repair. Finally the liberated DNA represents a building material for the biofilm matrix. The additional DNA binding

capacity of the pseudo type IV pili of the DNA uptake complex further promotes the formation of biofilm, encasing non-CSP responsive cells as well. In case that surrounding competitors are not sensitive to the released mutacins, autolysis represents a safe mechanism for liberating DNA that can be used as biofilm building material. Thus the phenotypic diversity and the altruistic behaviour of lytic cells provides benefit to the whole population. In summary, we performed a subpopulation specific transcriptome analysis comparing CSP induced competent and non-competent cells. The results show that flow cytometry and subsequent transcriptome analysis of *S. mutans* cells were successfully combined, resulting in a strong enrichment of mRNA of *comX* and its downstream targets, e.g. the transformasome related genes. We demonstrated for the first time, that bifurcation into competent and non-competent cells occurred at the level of ComE and not ComX, but that expression of bacteriocin related genes occurred similarly in all cells, particular the self acting mutacin CipB. We suggest that due to noise in the basal level of ComE expression some cells exceed a threshold of CSP activated ComE protein molecules, enabling a positive feedback loop and entering the competent state. Moreover, our data indicate a second bifurcation within the competent fraction, where some cells undergo autolysis presumably due to a decreased amount of CipI. Intra population diversity during competence development apparently has several advantages. It enables *S. mutans* to cope with competitors, produces membrane embedded DNA binding proteins, and liberates DNA that can be used for genetic diversity, repair or as biofilm building material, and thus reflects its successful adaptation to its ecological niche.

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CHAPTER V

The competence stimulating peptide inducible protein SpxB is a repressor of competence development in *Streptococcus mutans*

André Lemme and Irene Wagner-Döbler

Helmholtz-Centre for Infection Research, Research Group Microbial Communication, Inhoffenstr. 7, D-38124 Braunschweig

5 Chapter IV - The competence stimulating peptide inducible protein SpxB is a repressor of competence development in *Streptococcus mutans*

5.1 Abstract

Natural transformability is one of the key virulence determinants of *S. mutans*, since it allows the acquisition of fitness enhancing DNA from the environment. Moreover, competence initiation is coupled with bacteriocin production, facilitating autolysis and cell lysis of competitors. Increasing the basal level of competence e.g. by addition of the competence stimulating peptide (CSP) is a transient process and so far no mechanism for leaving the competent state has been presented.
Here we demonstrate that SpxB represses the expression of the alternate sigma factor ComX, which is responsible for the activation of late competence genes (DNA uptake and processing genes). Overexpression of SpxB decreased the number of transformants in the absence and presence of CSP, demonstrating its general capability in competence repression. Expression of SpxB was independent from ComDEX and it was not co-transcribed with adjacent late competence genes. We show that the histidine kinase HK9 is required to mediate the increased expression of *spxB* upon addition of CSP.
This study shows that SpxB is involved in the competence regulation of *S. mutans*. Its ability to interfere with ComX expression provides a general mechanism to leave the competent state, independently of the various upstream signals influencing ComX expression.

5.2 Introduction

Natural genetic transformation represents a mechanism of horizontal gene transfer and appears to be very widespread in bacteria (Johnsborg et al. 2007). Uptake of DNA requires the assembly of a multi-protein complex, that is produced in a physiological state termed competence.
Ccompetence is well studied in *Streptococcus mutans*, as a representative of the oral cavity and major etiological agent of human dental caries.
S. mutans uses several regulatory circuits, e.g. the ComCDE and the HdrRM system, to respond to different environmental stimuli. Both systems are partly membrane embedded and

transmit their cognate signals to the ComR/S system, which subsequent activates the alternate sigma factor ComX (Ahn et al. 2006, Mashburn-Warren et al. 2010, Okinaga et al. 2010b, Okinaga et al. 2010a). *S. mutans* exhibits a basal level of competence, which can be strongly enhanced through the addition of competence stimulated peptide (CSP), which is the processed gene product of *comC*. The two component system ComDE responds to the presence of CSP and is required for its signal transduction.
Expression of *comC* can be triggered when the cells are exposed to stressful conditions, e.g. low pH or antibiotics, thus connecting stress with competence development (Perry et al. 2009b). Increasing the level of competence by the addition of CSP in *S. mutans* is a transient process since it shuts down ~3-4 hours after its induction (Kreth et al. 2005). So far, no mechanism how *S. mutans* escapes from that raised level of competence has been described.
The Spx-like regulators are highly conserved in low-G+C content bacteria and several studies showed an involvement of Spx in the general stress (Nakano et al. 2003, Nakano et al. 2005, Pamp et al. 2006, Kajfasz et al. 2010). Zuber and co-workers reported that the C-terminal domain of the RNA polymerase (RNAP) alpha subunit (αCTD) is the primary target of Spx in *B. subtilis*. Interacting with the αCTD of the RNAP makes Spx a global transcriptional regulator that can positively or negatively influence transcriptional initiation (Nakano et al. 2010, Zuber 2004).
Recently, a Spx protein was shown to repress the induction of the early and late competence genes in *S. pneumoniae* under conditions that promote natural competence development through the accumulation of CSP (Turlan et al. 2009). Moreover a Spx protein suppressed the competence development in *B. subtilis* through transcriptional repression of the *srf* operon, and therefore preventing the expression of ComS. ComS antagonizes with the proteolytic complex of ClpC, the adapter protein MecA and the competence master regulator ComK and thus prevents ComK from proteolysis (Nakano et al. 2003). Moreover, Spx was shown to form a quaternary complex with ClpC, MecA and ComK and prevented the ComS mediated release of ComK (Nakano et al. 2002a, Turgay et al. 1997, Turgay et al. 1998) .
The activity of the Spx proteins can be regulated through proteolysis, since they are substrates of the ClpXP proteases (Kajfasz et al. 2009, Nakano et al. 2002b). Kajfasz *et al*. showed that inactivation of *clpP* or *clpX* resulted in an accumulation of the two Spx proteins in *S. mutans* (Kajfasz et al. 2009). Additional inactivation of *spxA* in Δ*clpP/X* backgrounds completely restored the observed growth defects, and impairment in long term survival and acid killing assays. Inactivation of *spxB* in Δ*clpP/X* resulted only in partial phenotypic recovery.
SpxB is located downstream of the two late competence genes *recA* and *cinA*, which are controlled by the sigma factor ComX, and was shown to be induced in the presence of CSP (Perry et al. 2009b), thus making it a possible regulatory element in the competence cascade of *S. mutans*. The scope of this study was to characterize the role of SpxB in the competence regulation of *S. mutans*. Using reporter strains and quantitative PCR we show that SpxB is a

repressor of the *com* genes. We tested different HK mutants and found one, that was responsible for the activation of SpxB in the presence of CSP.

5.3 **Results**

5.3.1 Deletion of SpxB increased expression of *com* genes.

To determine the influence of SpxB on competence development, we deleted the gene in the wildtype and two luciferase strains constructed for monitoring ComE and ComX expression. We found that in the presence of 0.2 µm CSP both promoters were induced significantly higher in the Δ*spxB* background, as shown with the luciferase reporter strains (Figure 37). Without addition of exogenous CSP, no difference in the promoter activity became apparent (data not shown).

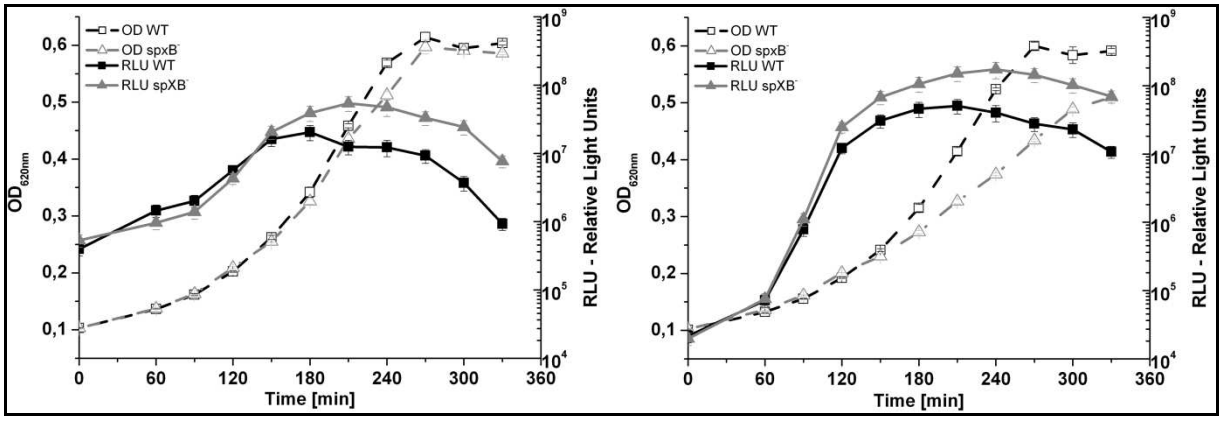

Figure 37. Deletion of *spxB* enhances transcription of *comE* and *comX*.
Growth characteristics and luminescence development of the *comE* (left) and *comX* (right) promoter-luciferase fusion in the wildtype and Δ*spxB* background in the presence of 0.2 µM CSP. CSP was added after one hour of incubation. Figures show the mean and standard deviation of at least two independent experiments.

To further confirm these data and to determine the influence on target genes of ComE and ComX, we extracted RNA at different time points after addition of CSP to the wildtype and the *spxB* mutant and performed quantitative real time PCR. The results are presented in figure 38. In contrast to the luciferase data, transcription of *comE* was not enhanced in the absence of the *spxB* gene, but transcription was extended for a longer time period. In the absence of *spxB*, transcription of *comX* was elevated at all tested time points, concluding that SpxB represses transcription of *comX* and presumably interferes with *comE* activation at later time

points. Similar to *comE*/*comX*, mRNA levels of *spxB* peaked two hours post induction but then decayed.

The ComE controlled genes *cipB*, *cipI* and *nlmA* were not affected by the lack of Δ*spxB* but transcription of *comYA* (late competence genes, directly activated by ComX) was enhanced at all tested time points, which is reasonable due to the elevated *comX* levels.

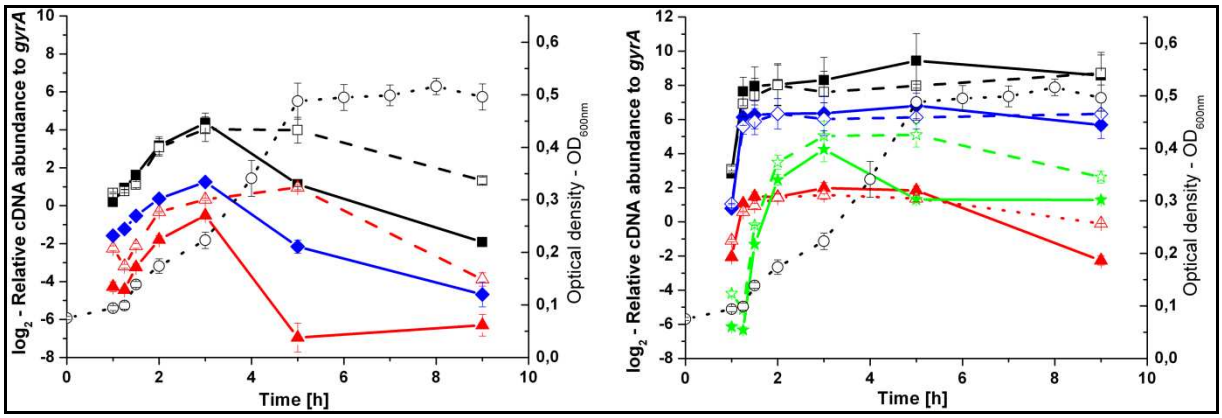

Figure 38. Effect of *spxB* deletion on the expression of competence related genes using qPCR.

The expression levels of target genes were normalized to *gyrA*. CSP (0.2 µM) was added after one hour of incubation. Prior to CSP addition, the samples for 0 min were withdrawn. Afterwards, sampling was carried out 15 min, 30 min, 1 h, 2h, 4 h, and 8 h post CSP-induction. The optical density of the wildtype strain is plotted to assign the different time points to the growth.

Left: Open circles and dotted line, optical density of the wildtype; squares, *comE* expression; triangles, *comX* expression; rhombus, *spxB* expression. Filled symbols and continuous line, wildtype; open symbols and interrupted line, *spxB* mutant. Right: Open circles and dotted line, optical density of the wildtype; squares, *cipB* expression; triangles, *cipI* expression; rhombus, *nlmA* expression; star, *comYA* expression. Filled symbols and continuous line, wildtype; open symbols and interrupted line, *spxB* mutant.

5.3.2 Overexpression of SpxB decreased ComX expression.

Since deletion of *spxB* resulted in elevated and prolonged transcription of *comX*, we were interested if overexpression would result in repression. To test this we cloned the *spxB* gene with an artificial ribosomal binding site into the pIB166 expression vector under the control of the P23 promoter (Biswas et al. 2008b) and transformed the plasmid into strain SMluccomX. Indeed, overexpression of SpxB resulted in a reduced luciferase activity for the *comX* gene compared to the empty vector control (Figure 39), confirming the role for SpxB to act as a repressor for competence gene induction. Since the activity of Spx proteins is controlled by the ClpPX proteases, attempts to overexpress Spx in *B. subtilis* failed due to rapid degradation of Spx by these proteases (Nakano et al. 2001, Nakano et al. 2002b). This might explain that the repression of *comX* was not so pronounced in the luciferase assay, since maintenance of high Spx protein levels is difficult due to rapid proteolysis .

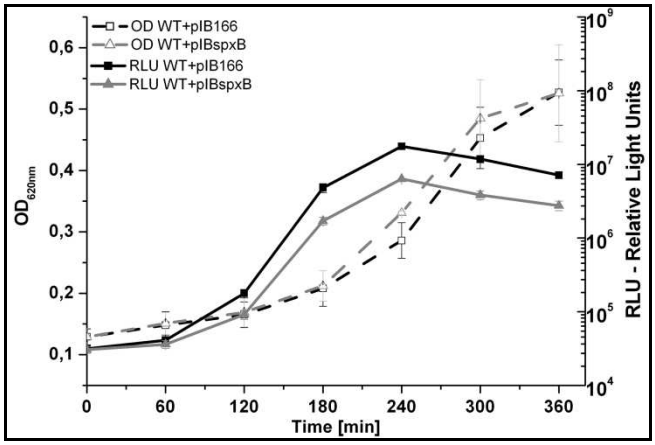

Figure 39. Repression of *comX* transcription by overexpression of SpxB.
Growth characteristics and luminescence development of the *comX* promoter-luciferase fusion in the wildtype carrying pIB166 (vector control) or pIBspxB (overexpression of spxB) in the presence of 0.2 µM CSP. CSP was added after one hour of incubation. Figures show the mean and standard deviation of two independent experiments.

5.3.3 Overexpression of SpxB decreased the transformation efficiency.

Deletion of *spxB* caused an up-regulation of *comX* and *comYA*. To see if increased expression of these genes correlates with transformation events, we measured the transformation rate of wildtype and Δ*spxB*. We did not observe any differences in the transformation capacity of both strains (in the presence and absence of CSP, data not shown). Thus, we assumed that the transcriptional induction of *comX* and *comYA* was not due to the recruitment of new cells becoming competent, but rather due to a higher transcription in the present competent cells through de-repression caused by the absence of SpxB. Therefore we tested the ability of the wildtype, carrying either pIB166 or pIBspxB, to take up and incorporate DNA. Indeed, higher expression of *spxB* resulted in significantly decreased transformation frequencies both in the absence and in the presence of exogenously added CSP (Figure 40), confirming finally on the phenotypic level the ability of SpxB to repress the competence development in *S. mutans*.

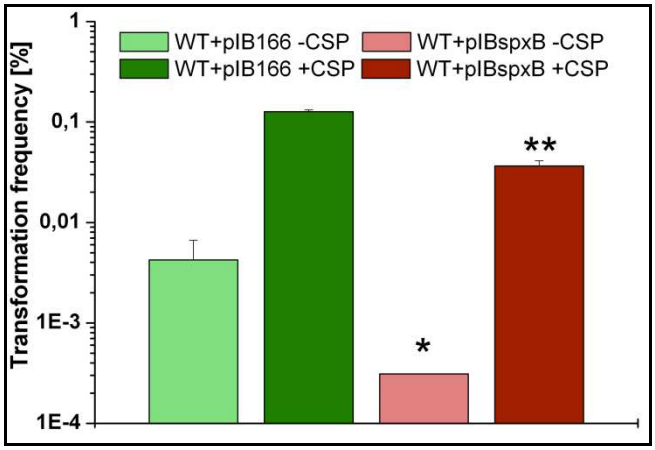

Figure 40. Overexpression of SpxB decreases transformation efficiency.

Transformation frequencies of *S. mutans* wildtype strain carrying pIB166 (vector control) or pIBspxB (overexpression of spxB) were determined in the absence and presence of CSP.
The error bars indicate standard deviations of 3 biological replicates. Significance was calculated using Student's t-test, comparing WT+pJB166 (light green bar) with WT+pIBspxB (light red bar), both without CSP (* $P < 0.05$), and the same strains, but with CSP (dark green and dark red bar, respectively; ** $P < 0.01$).

5.3.4 Regulation of the *spxB* gene – influence of CSP and acidic conditions.

To obtain more insights into the regulation of *spxB* we performed northern blotting with a probe for the full length *spxB* gene (399 nt). A band of ~ 500 bp became apparent in the wildtype but was missing in the knockout mutant (data not shown), Comparing non-induced and CSP treated cells, we (i) confirmed the up-regulation of *spxB* by CSP, (ii) showed that *spxB* was transcribed as a monocistronic mRNA and (iii) full length *spxB* was not co-transcribed with *recA* in the presence of CSP, due to the absence of additional bands in the CSP treated RNA populations (Figure 41a).
Since resolution in northern blots is low and primer extension experiments were not carried out, we cannot exclude that induction of *spxB* is driven from a second promoter, which is in close proximity to its housekeeping promoter and which is activated upon CSP addition.
To further confirm the lack of transcriptional read through, we carried out RT-PCR targeting full length transcripts of *spxB-recA* or *spxB-recA-cinA*, respectively (Figure 41b). The absence of PCR products confirmed that *spxB* is regulated independently from these late competence genes from its own promoter.
To determine if *spxB* is a target of the ComDEX pathway, we tested RNA of mutants for all three genes. All mutants gave the same pattern as the wildtype (Figure 41a), showing that *spxB* is activated independently of these *com* genes through a second CSP transduction pathway.
Perry and co-workers demonstrated, that transcription of *comC* could be induced, when cells were incubated at low pH (Perry et al. 2009b). We speculated, that if stress could increase the

transcription of *comC*, then *spxB* should be induced as well, due to the elevated presence of CSP. Indeed, a shift to pH 5.0 for two hours increased transcription of *spxB* ~3.3 fold, compared to the non-CSP control (Figure 41c.), demonstrating the connection between stress, competence and SpxB initiation, respectively.

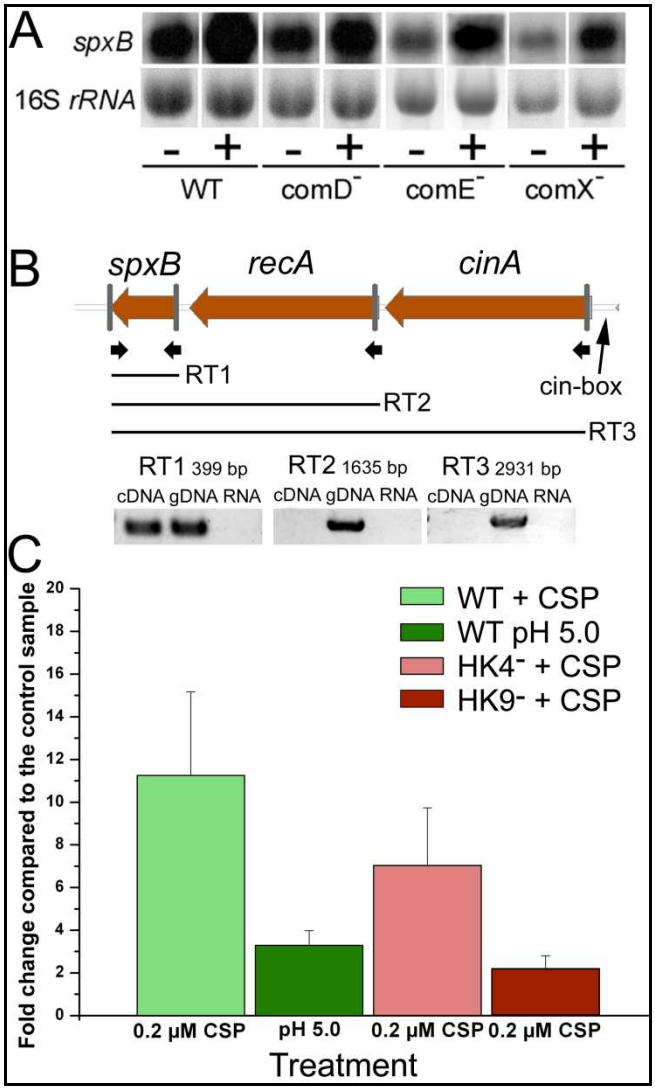

Figure 41. Regulation of *spxB* in the wildtype and various mutants.

A: Northern blot analysis for *spxB* in the wildtype and mutants defective in the *com* genes. Control samples (without CSP) are indicated with "minus". Samples marked with "plus", were incubated for 2 h in the presence of 0.2 µM CSP. The 16S rRNA served as loading control. **B**: RT-PCR of a CSP induced RNA sample of the population.
RT PCR was performed with three sets of primers. Genomic DNA (gDNA) and RNA served as positive and negative controls, respectively. **C**: Quantitative real time PCR of *spxB* in the wild type and two mutants defective for histidine kinases. Data represent the average fold change of two biological replicates, measured in duplicate. Control samples were incubated for two hours in the absence of CSP or without acidification, respectively.

5.3.5 Identification of an histidine kinase, needed for *spxB* induction.

Recently, Perry and co-workers determined the CSP regulon in *S. mutans* using microarray analysis. They found that in addition to ComDE two other TCS were activated through the presence of CSP, HK/RR4 and HK/RR9 (Perry et al. 2009b). Since the *comDEX* genes were dispensable for *spxB* activation, we were curious if the two CSP induced histidine kinases HK4 and HK9 were responsible for CSP recognition and subsequent *spxB* induction.
We incubated mutants, defective for both kinases, two hours in the presence of 0.2 µm CSP and compared the transcription of *spxB* with the non-CSP controls incubated for two hours, too (Figure 41c). The wildtype exhibited a ~11 fold up-regulation of *spxB*. A weaker, but still significant induction (~7 fold), was obtained in ΔSMU.928 (HK4). Deletion of SMU.1965 (HK9) resulted in almost no induction, suggesting that the histidine kinase HK9 was responsible for signal transduction and subsequent activation of SpxB, through its cognate response regulator.

5.4 Discussion

Interconnection of Spx proteins with Clp proteases and stress conditions.
The work of Kajfasz *et al.* showed that the Spx-like proteins in *S. mutans* are engaged in central parts of its metabolism, especially in processes crucial for adaptation to stress conditions, e.g. oxidative stress (Kajfasz et al. 2010). It was further shown that impaired phenotypes associated with mutation in the ClpXP proteases were due to accumulation of the Spx proteins (Kajfasz et al. 2010). Moreover they demonstrated that the role of SpxA was more important than that of SpxB to cope with different stresses, since even under non-stress conditions SpxA was indispensable for normal growth, whereas deletion of SpxB alone did not negatively influence the capability to withstand various stresses (Kajfasz et al. 2010). Similar observations were made in *S. pneumoniae*, were deletion of *spxA2* showed a strong growth defect but deletion of *spxA1* did not (Turlan et al. 2009), concluding that SpxB in *S. mutans* or SpxA1 in *S. pneumoniae* execute more specialised roles than their paralogues, respectively.
Here we demonstrate that, similar to SpxA1 in *S. pneumoniae* (Turlan et al. 2009), the SpxB protein of *S. mutans* is involved in the regulation of competence, acting as a repressor for expression of ComX and subsequent its downstream target genes.

Mechanism of regulator-RNAP interference by Spx proteins.

Transcriptional activation relies on interactions of promoter bound RNA-polymerase (RNAP) and DNA bound regulator/activator proteins. Crucial for most interactions is the α-subunit of the RNAP, since it provides several activator-interaction surfaces within the C-terminal domain (CTD) (Busby and Ebright 1999, Ishihama 1992). Zuber and co-workers demonstrated that Spx binds to the α-subunit of the RNAP and thus interferes with the interaction of activator proteins with the αCTD, leading to a reduced transcriptional initiation (Zuber 2004, Nakano et al. 2003).

A very important regulator of competence in *S. mutans* is ComR, since all signals (including CSP) which induce competence, are transmitted to ComR (Mashburn-Warren et al. 2010). ComR was shown to directly activate expression of ComX in *S. mutans* (Mashburn-Warren et al. 2010), independently of ComE. Our data show that SpxB is involved in the repression of ComX. Since expression of ComX requires the action of ComR, it might be possible that repression of ComR by SpxB, resulting in a reduced level of ComX, is the mechanism of SpxB mediated reduction in competence. The study of Chattoraj *et al.* (Chattoraj et al. 2010) provided an indirect evidence that decreased expression of ComR due to the action of SpxB is not likely since expression of both ComR (1.5 fold) and SpxB (1.8 fold) was increased in a *clpP* deletion mutant, suggesting that SpxB does not influence ComR expression. Since we observed a repression of ComX an interference of SpxB with the ComR-RNAP interaction, thereby inhibiting ComX expression, can be assumed but has to be determined experimentally. A similar mechanism has been found in *B. subtilis*, where the activator protein is the quorum sensing response regulator ComA, which is activated by its cognate histidine kinase ComP after exceeding a threshold level of the peptide ComX. Interaction of ComA with the αCTD of the RNAP was inhibited by Spx and thus prevented expression of the *srf* operon, harbouring *comS* (Zhang et al. 2006).

The role of the Spx protein in competence shutdown in *S. mutans* and other streptococci.

We report here, that the transcriptional activation of *spxB* in response to CSP is similar to that of *comE* and *comX*. High levels of CSP lead to a transient induction of competence beyond the CSP independent basal level. CSP dependent induction of competence implies not only an additional metabolic burden due to the production of competence proteins, it is also associated with cell lysis (Perry et al. 2009b). Thus it is reasonable that during activation of the *com* genes by CSP in *S. mutans* a repressor protein is induced for leaving the competent state. Inhibition of further transcriptional activation of the *com* genes by SpxB would permit proteases to clear the levels of competence proteins and to reset the system for initiation of a new round of competence.

In *S. pneumoniae*, spxA1 was found to suppress competence, but it is not known how it is regulated (Turlan et al. 2009). According to a microarray study carried out by Peterson *et al*., the addition of CSP to *S. pneumoniae* did not increase the expression of one of its two Spx

homologues (Peterson et al. 2004). Since CSP is not the signal triggering *spxA1* expression, it will be of interest to determine under which conditions *spxA1* is expressed or if the basal activity is sufficient to repress the *com* genes in *S. pneumoniae*.
Consideration of the genome sequence suggests that *S. gordonii* uses the same competence regulatory cascade as *S. pneumoniae* (Martin et al. 2006). *S. gordonii* harbours four Spx like proteins, of which one is located downstream of *recA/cinA* like in *S. mutans*. Interestingly, this gene was induced upon CSP addition (Vickerman et al. 2007b), suggesting a similar role for Spx in *S. gordonii*. Moreover, the arrangement of *spx-recA-cinA* is frequently encountered in the genus Streptococcus, e.g. in the pyogenes group (i.e. *S. pyogenes* and *S. agalactiae*), in the mitis group (i.e. *S. gordonii* and *S. sanguninis*) or within the bovis group (i.e. *S. thermophilus*), suggesting a conserved mechanism for leaving competence in streptococci.

The role of the histidine kinase HK9.
We show that in *S. mutans*, deactivation of HK9 greatly reduced the transcriptional induction of *spxB* in the presence of CSP, suggesting that HK9 may be involved in CSP signal transduction. The work of Biswas *et. al* and Levesque *et al*. revealed that HK9 does not seem to be involved in responding to various stresses but its inactivation resulted in a reduced transformation rate both in the absence and presence of CSP (Biswas et al. 2008a, Levesque et al. 2007). The reduced transformation rate and the lost ability to induce *spxB* in the presence of CSP of the HK9 deletion mutant implies a dual role for HK9, which is involved in establishing competence through a yet unknown mechanism and in leaving the competent state through the activation of *spxB.*

Conclusion

In summary, we show that the Spx homologue SpxB in *S. mutans* is a repressor of the alternate competence sigma factor ComX and that overexpression decreased the number of transformants in the absence and presence of CSP, demonstrating that SpxB represses competence independently of the various signals upstream of ComX. SpxB was also regulated independently from ComDEX. Expression occurred as a monocistronic mRNA and there was no transcriptional read-through with the upstream located late competence genes *recA/cinA*. The histidine kinase HK9 was shown to mediate the CSP dependent induction of SpxB. Addition of CSP to *S. mutans* not only raises the number of cells becoming competent it also stimulates the expression of *spxB*, and thus provides a mechanism that allows the escape from the competent state.

Acknowledgement

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6 Summary

Characterization of MleR

1. The previously described transcriptional activation of *mleR* by AI-2 could not be confirmed using quantitative PCR and a luciferase reporter strain measuring the promoter activity of *mleR*.
2. Sequence homology suggested that MleR might regulate the adjacent malolactic fermentation gene cluster and, and thus it may be involved to confer resistance to acid stress in *S. mutans*. This role was investigated in detail and it was shown that the *mle* genes were induced upon acid shock within 30 minutes, demonstrating that malolactic fermentation is part of the early acid tolerance response in *S. mutans*.
3. It was shown that MleR regulates positively the expression of its own and the *mle* genes, respectively.
4. The strongest effect of MleR mediated gene expression was observed in the presence of L-malate, demonstrating that it is used as co-inducer of MleR.
5. Electrophoretic mobility shift assays confirmed the role of L-malate as a co-inducer since it altered the DNA binding capacities of MleR to the promoter regions of the *mle* genes.
6. It was shown that MLF highly contributed to *S. mutans* aciduricity when the cells were grown at a lethal pH value of pH 3.1, since the survival was increased more than one order of magniture.

Genetic, physiological and chemical complementation of a *luxS* mutant of *S. mutans*

7. During complementation of the $luxS_1$ mutant, secondary mutations were identified that were responsible for the observed phenotypes previously published.
8. Construction of a new *luxS* mutant revealed that deletion of *luxS* had no influence on important virulence traits of *S. mutans* (acid tolerance, biofilm formation, mutacin production). Transcriptome analysis showed that mutation of *luxS* affected the expression of genes involved in the glutamate and sulphur *m*etabolism but in a subtle way that did not affect growth or virulence.
9. Introduction of the SAH-hydrolas*e (sahH*) to complement the metabolic defects of a *luxS* mutation restored the expression of the genes involved in glutamate and sulphur metabolism beyond wildtype levels and thus showed that these changes were due to the metabolic disruption of the AMC rather than to AI-2 signalling.
10. Introduction of *sahH* also caused a number of other changes, i.e. altered biofilm structure and acid tolerance, which were also reflected in large changes in the transcriptome. Thus, this strategy does not overcome the problem of separating the metabolic changes of a *luxS* mutation from its signalling role.
11. Addition of AI-2 at various concentrations had no influence on any of the tested phenotypes and the transcriptome of the *luxS* mutant expressing *sahH*, indicating that

AI-2 does not play a role as a signalling molecule for *S. mutans* under the conditions used.

Single cell analysis of CSP mediated competence development in *S. mutans*.

12. Segregation into competent and non-competent cells of a clonal population was visualized on the single cell level using a GFP reporter for ComE, the response regulator that is activated by CSP and Com X, the alternate sigma factor. The data showed that bifurcation already occurs on the level of ComE.
13. The *comX* induced subpopulation was separated from the non-induced cells guided by the *comX* reporter using flow cytometry.
14. Microarray analysis of both subpopulations was successfully carried out using $3 \cdot 10^7$ cells of each fraction. A strong enrichment of all known regulatory elements of competence development and the complete transformasome genes for uptake and processing of DNA was achieved in the competent fraction as expected.
15. Interestingly, expression of the mutacin and immunity genes was similar in all cells with one exception, the protein that confers immunity to mutacin V, which was enriched in the competent subpopulation. This observation is in contrast to the current hypothesis that all competent cells undergo autolysis due to the intracellular accumulation of mutacin V.
16. Using fluorescence microscopy and counterstaining with PI we showed that a fraction of competent cells indeed triggered autolysis in an early stage of competence development since the level of GFP was low. The majority of competent cells was viable, showing that the competent subpopulation itself bifurcates into two subpopulations.
17. Visualization of DNA uptake on the single cell level confirmed that CSP activated competent cells were able to internalise DNA.

Characterization of SpxB and its role in competence development

18. SpxB belongs to the family of Spx proteins which had been shown to be involved in the competence regulation of *B. subtilis* and *S. pneumoniae*. Therefore, the influence of SpxB in the competence development of *S. mutans* was investigated and revealed that SpxB is a repressor of competence and presumably interferes with the ComR mediated activation of *comX*.
19. SpxB was shown to be inducible by CSP but its induction was dependent on the histidine kinase HK9 and not on the known CSP receptor ComD.

7 Material and methods

Table 7. Strains for characterization of MleR

Strain	Relevant characteristics	Reference/source
E. coli		
DH5α	General cloning strain	
Tuner(DE3)	Expression strain	Novagen
S. mutans		
UA159	Wild-type, Erm^s, Sp^s	ATCC 700610
ALSM03	UA159ΔmleR, Erm^r	This study
ALSM20	UA159::ϕ($mleR_P$-luc), Sp^r	This study
ALSM13	UA159ΔmleR::ϕ($mleR_P$-luc), Erm^r, Sp^r	This study
ALSM33	UA159::ϕ($mleS_P$-luc), Sp^r	This study
ALSM34	UA159ΔmleR::ϕ($mleS_P$-luc), Erm^r, Sp^r	This study

Table 8. Plasmids for characterization of MleR

Plasmid	Relevant characteristics	Reference/source
pFW5	Suicide vector, Sp^r	(Podbielski et al. 1996)
pHL222	Ap^r, luc	H. Lössner
pALEC15	Derivate pFW5, luc, Sp^r	This study
pALEC16	pALEC15 + ϕ(*mleR*$_P$-luc), Sp^r	This study
pALEC47	pALEC15 + ϕ(*mleS*$_P$-luc), Sp^r	This study
pALEC30	pET28c + ϕ(*mleR*$_{CDS}$), Km^r	This study

Table 9. Primers used for characterization of MleR

Primer[a]	Sequence (5'→3')	Purpose
135UpF	CCAAATAACCCGCATATTGAGG	Knockout *mleR*
135UpR	**GGCGCGCC**TTGAAATTTTTCAGCAACCTTA	Knockout *mleR*
135DoF	**GGCCGGCC**TCCTCAACCTTAACACCTGATA	Knockout *mleR*
135DoR	GTTGCTAAAGATTTGTTCTCAG	Knockout *mleR*
ErmF	**GGCGCGCC**CCGGGCCCAAAATTTGTTTGAT	ErmEA
ErmR	**GGCCGGCC**AGTCGGCAGCGACTCATAGAAT	ErmEA
lucF	ATATA**CCATGG**AAGACGCCAAAAAC	Luciferase
lucR	AAAAAA**ACTAGT**TTATGCTAGTTATTGCTCAGCGG	Luciferase
P135F/EP9	AAAAAA**CCATGG**CTTTATTCAAAAAAGGATCGTTT	Promoter *mleR*/EMSA
P135R	TTTTTT**CCATGG**TTAACCTTTCTATTATTTTTACTAGTT	Promoter *mleR*
P137F/EP6	AAATTT**CCATGG**CAAGACTGTTAAAGTCAAAAA	Promoter *mleS*/EMSA
P137R/	AAAAAA**CCATGG**TTTCTGCACCTCCTTATATT	Promoter *mleS*
135qF	TGAAGCGTCACCTTGAGAGA	SMU.135 QPCR
135qR	TAATGGGTGGGCATCCTAAG	SMU.135 QPCR

136qF	AAGGTATCATCGGCAAGCAC	SMU.136 QPCR
136qR	TCACTTTTTCAAGCGTCTGC	SMU.136 QPCR
137qF	GGTATCTTTGCGGCTATGGA	SMU.137 QPCR
137qR	TTTCACGCAAGACACGAGAG	SMU.137 QPCR
138qF	CGACGGATAGCAAGTCTGGT	SMU.138 QPCR
138qR	GTCAACGTGCTAGTCGCAAA	SMU.138 QPCR
139qF	TACAGCGATTGACGAGAACG	SMU.139 QPCR
139qR	AGAAATTGGCTTCGCTGAAA	SMU.139 QPCR
140qF	TTCCTATGCGGATTTTCAGG	SMU.140 QPCR
140qR	CCTGACCGATTTGGGAATA	SMU.140 QPCR
1114qF	TACTACCCGGCCCCGATT	SMU.1114 QPCR
1114qR	CGAGCACGCAAAACAATAGA	SMU.1114 QPCR
EP1	TTAACCTTTCTATTATTTTTACTAGTT	EMSA
EP2	TCCAAGTGGTTTAAAAGTAACAAGA	EMSA
EP3	GCAACTTCCCAAGAGAAAACA	EMSA
EP4	TTAATCAAGATTATCAATAATCTC	EMSA
EP5	ATGAAGAAAAAAAGCTATCT	EMSA
EP7	TGCTTGCCGATGATAGGTT	EMSA
EP8	TAAAGAATACAAGTTTAAAAGCAAATAGTTAACT	EMSA
EP10	ATAAGTATTTTTTATCCGTTATCTAAGGTTTGAC	EMSA
EP11	GTCAAACCTTAGATAACGGATAAAAAATACTTAT	EMSA

Restriction sites in bold

Table 10. Strains for complementation of the *luxS* mutation

Strain	**Relevant characteristics**	**Reference/source**
E. coli		
DH5α	General cloning strain	
V. harveyi		
BB170	*luxN*::Tn*5*; AI-1 positive; AI-2 positive, Km^r	ATCC BAA-1117
S. mutans		
UA159	Wild-type, Erm^s, Sp^s	ATCC 700610
WT+pDL278	UA159, carrying pDL278, Erm^s, Sp^r	This study
WT+pDL*luxS*	UA159, carrying pDL*luxS*, Erm^s, Sp^r	This study
Δ*luxS*$_1$	UA159Δ*luxS*, Erm^r	(Sztajer et al. 2008)
Δ*luxS*$_2$	UA159Δ*luxS*, Erm^r	This study
Δ*luxS*+pDL278	UA159Δ*luxS*, carrying pDL278, Erm^r, Sp^r	This study
Δ*luxS*$_2$pDLluxS	UA159Δ*luxS*, carrying pDL*luxS*, Erm^r, Sp^r	This study
Δ*luxS*$_2$SahH	UA159Δ*luxS*::ϕ(*aad9*$_p$-*sahH*, *aad9*$_p$-*aad9*), Sp^r	This study
ΔnlmA	UA159Δ*nlmA*, Erm^r	Reck, unpublished
ΔcipB	UA159Δ*cipB*, Erm^r	Reck, unpublished

Sp^r, spectinomycin resistance; Erm^r, erythromycin resistance; Km^r, kanamycin resistance; sahH, S-adenosylhomocysteine hydrolase;

Table 11. Plasmids used for complementation of the *luxS* mutation

Plasmids	Relevant characteristics	Reference/source
pFW5	Suicide vector, Sp^r	(Podbielski et al. 1996)
pDL278	*E. coli-Streptococcus* shuttle vector, Sp^r	(Dunny et al. 1991)
pDLluxS	pDL278 + ϕ(*luxS*$_p$-*luxS*), Sp^r	This study
pFW5-sahH	pFW5+ ϕ(*aad9*$_p$-*sahH*, *aad9*$_p$-*aad9*), Sp^r	This study

Table 12. Primers used for complementation of the *luxS* mutation

Primer	Sequence (5'→3')	Purpose
P1luxS	CTGATAAAACAACCACCTCTGG	LuxS Upstream
P2luxS	**GGCGCGCC**TGAAATAAGACGGACATAAGGG	LuxS Upstream
P3luxS	**GGCCGGCC**TCAGCTAAAGAATGGGCAAAGC	LuxS Downstream
P4luxS	GACTCCAATACACCAGCTAATGGC	LuxS Downstream
ErmF	**GGCGCGCC**CCGGGCCCAAAATTTGTTTGAT	Erm
ErmR	**GGCCGGCC**AGTCGGCAGCGACTCATAGAAT	Erm
P1nlmA	TTCCCCGCCACTACTAAACA	NlmA Upstream
P2nlmA	**GGCGCGCC**AATGAAAGTGTTTGGCTGTCCA	NlmA Upstream
P3nlmA	**GGCCGGCC**AGGCACCCATATATTCTTGCTG	NlmA Downstream
P4nlmA	TTGAGACGTACCACCACTGC	NlmA Downstream
P1cipB	ACTGCCTGAGATGGAGTTGC	CipB Upstream
P2cipB	**GGCGCGCC**TCGTTAAATTGTTCAAATGCTTG	CipB Upstream
P3cipB	**GGCCGGCC**CGGAGGAGCTCTTAATTCCTG	CipB Downstream
P4cipB	TCTTTTTGTTTTAGAAACTTCTGCT	CipB Downstream
PluxSF	AAAAATA**GTCGAC**ATTGTTCTTTGCTGCTTAGAT	pDL*luxS*
CDSluxSR	AAAAATT**GTCGAC**TTACACTAGATGACGCTCAAA	pDL*luxS*
UpluxSF	AAAAAAA**GCTAGC**GTCAAGCTCTCAAGCGTTCGGATA	LuxS upstream
UpluxSR	AAAAAAA**GCTAGC**AACAGTAACTTCTTTTGTCAT	LuxS upstream
PaadFor	TTTTT**GGATCC**TGATTTTCGTTCGTGAATACATG	Paad
PaadRev	AAAAAT**GGATCC**TCCTCACTATTTTGATTAGTAC	Paad
SahHFor	TTTTTTT**CTCGAG**ATGTTTTTTAAGGAGATCGTGATGA	SahH
SahHRev	TTTTTTT**CTCGAG**GTTTAAGAAAAGGGCAGCCTAT	SahH
DoluxSF	TAA**CCATGG**CCTTTTGAGCGTCATCTAGTG	LuxS Downstream
DoluxSR	TAAAAAAA**CCATGG**CTTAAGGACAGCAAATATTATGACGAGG	LuxS Downstream
1114qPCRF	TACTACCCGGCCCCGATT	qPCR
1114qPCRR	CGAGCACGCAAAACAATAGA	qPCR
137qPCRF	GGTATCTTTGCGGCTATGGA	qPCR
137qPCRR	TTTCACGCAAGACACGAGAG	qPCR
150qPCRF	TTGAAGGTGGGAAGGTATCG	qPCR
150qPCRR	ATCGAATGAGTCCCCAAGTG	qPCR
1004qPCRF	CATACAGTAACGACAAGCAGTAGCTCTA	qPCR
1004qPCRR	GTACGAACTTTGCCGTTATTGTCATA	qPCR
1005qPCRF	GACAACACCTTACTTCCTAAATCG	qPCR
1005qPCRR	GCTTGGTTACCACTCGTTGAATAA	qPCR
1895qPCRF	ATAGAAGGCGGCATGACTTG	qPCR
1895qPCRR	AAGGAACAATAACAGGATTTGGA	qPCR
1957qPCRF	GAAGAAGCGCGTTCTAATGG	qPCR
1957qPCRR	GTCCAAGAGCAATTCCCAAA	qPCR

Restriction sites in bold

Table 13. Strains for subpopulation specific transcriptome analysis

Strain	Relevant characteristics	Reference/source
E. coli		
DH10β	General cloning strain	New England Biolabs
S. mutans		
UA159	Wild-type, Erm^s, Sp^s	ATCC 700610
SMluccomE	UA159::ϕ(*comE*$_P$-luc), Sp^r	This study
SMluccomX	UA159::ϕ(*comX*$_P$-luc), Sp^r	This study
SMGFPcomX	UA159, carrying pALSM04	This study
SMGFPcomE	UA159, carrying pALSM28	This study
SMGFPcipB	UA159, carrying pALSM34a	This study
ΔcomD	UA159Δ*comD*, Erm^r	(Levesque et al. 2007)
ΔcomE	UA159Δ*comE*, Erm^r	(Perry et al. 2009b)
ΔcomX	UA159Δ*comX*, Erm^r	M. Reck, unpublished
ΔHK4	UA159ΔSMU.928, Erm^r	(Levesque et al. 2007)
ΔHK9	UA159ΔSMU.1965, Erm^r	(Levesque et al. 2007)

Erm, erythromycin; Sp, spectinomycin

Table 14. Plasmids for subpopulation specific transcriptome analysis

Plasmid	Relevant characteristics	Reference/source
PKRC12	Donor of eGFP	(Riedel et al. 2001)
pFW5	Suicide vector, Sp^r,	(Podbielski et al. 1996)
pALEC15	Derivate pFW5, luc, Sp^r	(Lemme et al. 2010)
pALEC50	pALEC15 + ϕ(*comE*$_P$-luc), Sp^r	This study
pALEC52	pALEC15 + ϕ(*comX*$_P$-luc), Sp^r	(Kunze et al. 2010)
pALSM01	modifed pAT18, Erm^r	(Nakagawa et al. 2001, Trieu-Cuot et al. 1991)
pALSM02	pALSM01, eGFP, Erm^r	This study
pALSM03	pALSM03, Removal of lac_p, Erm^r	This study
pALSM04	pALSM03 + ϕ(*comX*$_P$-eGFP), Erm^r	This study
PALSM28	pALSM03 + ϕ(*cipB*$_P$-eGFP), Erm^r	This study
PALSM34a	pALSM03 + ϕ(*comE*$_P$-eGFP), Erm^r	This study

Erm, erythromycin; Sp, spectinomycin; Cm, chloramphenicol; tetracycline; luc, luciferase

Table 15. Primer for subpopulation specific transcriptome analysis

Primer[a]	Sequence (5'→3')	Purpose
lucF	ATATA**CCATGG**AAGACGCCAAAAAC	Luciferase
lucR	AAAAAA**ACTAGT**TTATGCTAGTTATTGCTCAGCGG	Luciferase
PcomEF	TTTTTT**CCATGG**GGGTGTCGTCATTCTTCCTT	pALEC50
PcomER	TTTTTT**CCATGG**TATTTCTCCTTTAATCTTCTAT	pALEC50
PcomXF	AAAAAAA**CCATGG**TCCAAAAATAAGTGACTAAGG	pALEC52
PcomXR	AAAAAAA**CCATGG**CTATTACGATGACCTCCTTT	pALEC52
GFPF	AAAAAA**TCTAGA**AA**GATATC**ATGAGTAAAGGAGAAGAACTT TTCACTGG	eGFP

GFPR	AAAAAA**GCTAGC**CTATTTGTATAGTTCATCCATGCCATG	eGFP
PcomX2F	TCCAAAAATAAGTGACTAAGG	Promoter comX
PcomX2R	CTATTACGATGACCTCCTTT	Promoter comX
PcipBF	TTTATATCTCCTTTTTTTGATTATAATTAGTATACTA	Promoter cipB
PcipBR	ATGATAAATACCCCTTCCCCATTTTTAGTT	Promoter cipB
PcomE2F	TTATATAATCAATTGACAACGGCTGAT	Promoter comE
PcomE2R	TATTTCTCCTTTAATCTTCTAT	Promoter come
QPCRcomRF	TATTACGAACGCCAACCTAT	QPCR
QPCRcomRR	TTCTTCTTCAGGCAAATCAT	QPCR
qPCRcomSF	TCAAAAAGAAAGGAGAATAACA	QPCR
qPCRcomSR	TCATCTGACATAAGGGCTGT	QPCR

Table 16. Strains for characterization of SpxB in its role in competence development

Strain	**Relevant characteristics**	**Reference/source**
E. coli		
DH10β	General cloning strain	New England Biolabs
S. mutans		
UA159	Wild-type, Erm^s, Sp^s	ATCC 700610
Δ*spxB*	Δ*spxB*, Erm^r, Sp^s	This study
SmluccomE	UA159::ϕ(*comE*$_P$-luc), Sp^r	This study
Δ*spxB*luccomE	Δ*spxB*::ϕ(*comE*$_P$-luc), Erm^r, Sp^r	This study
SmlucocmX	UA159::ϕ(*comX*$_P$-luc), Sp^r	This study
Δ*spxB*luccomX	Δ*spxB*::ϕ(*comX*$_P$-luc), Erm^r, Sp^r	This study

Erm, erythromycin; Sp, spectinomycin; luc, luciferase

Table 17. Plasmids for characterization of SpxB in its role in competence development

Plasmid	**Relevant characteristics**	**Reference/source**
pFW5	Suicide vector, Sp^r,	(Podbielski et al. 1996)
pALEC15	Derivate pFW5, luc, Sp^r	(Lemme et al. 2010)
pALEC50	pALEC15 + ϕ(*comE*$_P$-luc), Sp^r	This study
pALEC52	pALEC15 + ϕ(*comX*$_P$-luc), Sp^r	(Kunze et al. 2010)
pIB166	shuttle vector, Cm^r	(Biswas et al. 2008b)
pIB166spxB	pIB166::spxB$_{full\ length}$, Cm^r	This study
pLSE4	Tc^r	(Diaz and Garcia 1990)
pVA-GTFA	Erm^r	(Li et al. 2001b)

Erm, erythromycin; Sp, spectinomycin; Cm, chloramphenicol; Ap^r, Ampicillin; Tc^r,

Table 18. Primers used for characterization of SpxB in its role in competence development

Primer	**Sequence (5'→3')**	**Purpose**
spxBupF	AATCTTCTGGTAAGACAACTGTCGCT	Upstream spxB
spxBupR	**GGCGCGCC**CTTTCTTACAGCTTGTACAGCTTGAAA	Upstream spxB

spxBdoF	**GGCCGGCC**TTGAAAACACAGCAGCTCGTTTA	Downstream spxB
spxBdoR	CAGATGCAAGTTTGATGGCAAA	Downstream spxB
ermF	**GGCGCGCC**CCGGGCCCAAAATTTGTTTGAT	Erm cassette
ermR	**GGCCGGCC**AGTCGGCAGCGACTCATAGAAT	Erm cassette
lucF	ATATA**CCATGG**AAGACGCCAAAAAC	Luciferase
lucR	AAAAAAA**ACTAGT**TTATGCTAGTTATTGCTCAGCGG	Luciferase
PcomEF	TTTTTT**CCATGG**GGGTGTCGTCATTCTTCCTT	Construction pALEC50
PcomER	TTTTTT**CCATGG**TATTTCTCCTTTAATCTTCTAT	Construction pALEC50
PcomXF	AAAAAAA**CCATGG**TCCAAAAATAAGTGACTAAGG	Construction pALEC52
PcomXR	AAAAAAA**CCATGG**CTATTACGATGACCTCCTTT	Construction pALEC52
spxBCDSF	GAGGAGGTAAATGATTAAAATTTATACAATTTCAAGCTGT	CDS spxB
spxBCDSR	TTATAAAGCTGCCCGTAAACGA	CDS spxB
QPCRgyrAF	TACTACCCGGCCCCGATT	QPCR
QPCRgyrAR	CGAGCACGCAAAACAATAGA	QPCR
QPCRcomEF	GGACGTCTTGAAACCACCAT	QPCR
QPCRcomER	CCTTTTCAGGGATAGCGTCA	QPCR
QPCRcomXF	CTGTTTGTCAAGTGGCGGTA	QPCR
QPCRcomXR	GCATACTTTGCCTTCCCAAA	QPCR
QPCRcipBF	TTGTGCAGCAGGTATTGCTC	QPCR
QPCRcipBR	AAGAGCTCCTCCGATTCCTC	QPCR
QPCRcipIF	AAATCTCCTCTGCTTGTTCA	QPCR
QPCRcipIR	AAAAGCAAAAACACGACAGT	QPCR
QPCRnlmAF	TTGAAGGTGGGAAGGTATCG	QPCR
QPCRnlmAR	ATCGAATGAGTCCCCAAGTG	QPCR
QPCRcomYAF	CTTTTTTCTGGACGTCACGATTT	QPCR
QPCRcomYAR	TCGCCCCTTGATTTCATTTAAA	QPCR
QPCRspxBF	AGAAAATGGAATTGAAAGCA	QPCR
QPCRspxBR	CCTATCTGAAGACGCTTGTC	QPCR

Restriction sites in bold

7.1 Bacterial cultivation

7.1.1 Media

Media were obtained from the companies listed below. Chemicals were purchased from Sigma, Merck or Carl-Roth, respectively.

Agar agar	Bacto
Brain Heart Infusion	Bacto
Luria Bertani	Roth
Todd Hewitt Broth	Bacto
Tryptone	Bacto
Yeast extract	Bacto
Casamino acids	Bacto

7.1.1.1 Todd Hewitt Broth medium + yeast extract (THBY)

For preparation of THBY medium, 30 g Todd Hewitt Broth and 5 g yeast extract were dissolved in 1 l of deionised water. For solid media, 20 g/l or 10 g/l (for preparation of overlay agar) agar was added. The medium was autoclaved at 110°C for 10 minutes.

7.1.1.2 Brain Heart Infusion medium (BHI)

For preparation of BHI medium, 37 g Brain Heart Infusion were dissolved in 1 l of deionised water. For solid media, 20/l g agar was added. The medium was autoclaved at 121°C for 15 minutes.

7.1.1.3 Biofilm Medium (BM)

The basis solution of BM contained:

- 58 mM K_2HPO4
- 15 mM KH_2PO_4
- 10 mM $(NH_4)_2SO_4$
- 35 mM NaCl.

The solution was autoclaved at 121°C for 15 minutes. Prior use, the following components (0.2 µm filter sterilized, stored at -20°C) were added (end concentration):

- 0.2% (w/v) casamino acids
- 0.04 mM nicotinic acid
- 0.1 mM pyridoxine HCl
- 0.01 mM pantothenic acid
- 1 µM riboflavin
- 0.3 µM thiamine HCl
- 0.05 µM D-biotin
- 4 mM L-glutamic acid
- 1 mM L-arginine HCl
- 1.3 mM L-cysteine HCl
- 0.1 mM L-tryptophan

- 2 mM MgSO4 · 7H2O
- 100 µM MnCl2 · 4H2O

7.1.1.4 Autoinducer bioassay medium (AB)

The basis solution contained:

- 0.3 M NaCl
- 0.05 M $MgSO_4$

The pH was set with 0.1 M KOH to pH 7.5. The solution was autoclaved at 121°C for 15 minutes. For solid media, 20 g/1 agar was added.

Prior use, the following 0.2 µm filtered components were added to 1 l of the basis solution:

- 10 ml 1 M potassium phosphate buffer (pH = 7.0)
- 10 ml 0.1 M L-arginine
- 20 ml 50% glycerol
- 20 ml 10% (w/v) casamino acids.

Liquid media was supplemented with a sterile solution of trace elements and vitamins according to Vilchez *et al.* (Vilchez et al. 2007), containing: 7.4 µM KI; 19 mM $MgCl_2$-hydrat; 255 µM $ZnCl_2$; 694 µM $FeCl_3$-6-hydrat; 43 µM $CuCl_2$-2-hydrat; 118 µM $MnCl_2$-4-hydrat; 10 µM $(NH_4)_6Mo_7O_{24}$-4-hydrat; 110 mM $CaCl_2$-hydrat; 2 µM Na_2SeO_4; 30 µM $CrCl_3$-6-H_2O; 23 µM retinol; 14 µM β-carotene; 0.13 µM cholecalciferol; 0.7 µM vitamin K1; 0.2 µM (+)-α-tocopherol; 0.04 µM thiamine hydrochloride; 0.04 µM (−)-riboflavin; 0.1 µM pyridoxine hydrochloride; 0.007 µM vitamin B12; 1.5 µM nicotinic acid; 0.3 µM pantothenicacid hemicalcium salt; 4.5 µM folic acid; 0.6 µM, biotin and 340 µM L-ascorbic acid.

7.1.1.5 Luria-Bertani medium (LB)

For preparation of LB media, 25 g LB or 40 g LB-agar were dissolved in 1 l of deionised water for liquid or solid media respectively. The medium was autoclaved at 121°C for 15 minutes.

7.1.1.6 SOC medium

SOC medium contained:

- 0.5% (w/v) yeast extract
- 2.0% (w/v) tryptone
- 10 mM NaCl
- 2.5 mM KCl
- 10 mM $MgCl_2$
- 10 mM $MgSO_4$

The media was autoclaved at 121°C for 15 minutes. Afterwards 20 mM D-glucose (0.2 µm filtered) was added.

7.1.1.7 TSS medium

For preparation of TSS medium LB media was supplemented with:

- 10% (w/v) PEG 8000
- 30 mM $MgCl_2$
- 5% (v/v) DMSO.

The medium was autoclaved at 121°C for 15 minutes.

7.1.1.8 Antibiotics

Antibiotic stock solutions were dissolved in their respective solvent, 0.2 µm filtered, stored at –20°C and diluted prior use in the growth media.

Table 19. Antibiotics used in this study.

Antibiotic	Solvent	Stock concentration [mg/ml]	Working concentration *E. coli* [µg/ml]	Working concentration *S. mutans* [µg/m]
Ampicillin	water	100	100	-
Chloramphenicol	70% (v/v) ethanol	20	20	20
Erythromycin	70% (v/v) ethanol	50	200	4, 10
Spectinomycin	water	100	50	500
Tetracycline	methanol	25	25	20

7.1.1.9 Cultivation conditions

Liquid cultures of *E. coli* and *S. mutans* were both inoculated from glycerol stock cultures. Glycerol stock cultures of *V. harveyi* were streaked on agar plates and incubated overnight at 30°C, prior the inoculation of liquid media.
E. coli was routinely cultivated in LB media at 37°C with agitation (180 rpm). *V. harveyi* strains were routinely cultivated in AB media at 30°C in baffled flasks with agitation (180 rpm). *S. mutans* strains were grown at 37°C without agitation aerobically (5% CO_2 enriched) or under anaerobic conditions (80% N_2, 10% H_2, 10%). Anaerobic conditions were routinely used for BM and CDM media.

7.1.1.10 Glycerol stock cultures

For preparation of glycerol stock cultures, cells were grown in the respective media supplemented with the appropriate antibiotic. In the mid-log phase 25 ml cells were harvested and resuspended in 4 ml of fresh media and 2 ml 87% (v/v) glycerol, aliquoted and stored at –70°C.

7.1.2 Autoinducer-2 bioassay

The autoinducer-2 bioassay were carried out according to Vilchez *et al.* (Vilchez et al. 2007). A glycerol stock culture of *V. harveyi* BB170 was streaked on AB agar plates and incubated

overnight at 30°C. Bacteria were scrapped from the plate and resuspended in AB media. The cell suspension was diluted with AB to an optical density of ~0.5-0.7 and further incubated. After reaching an OD of 1.0, cells were diluted 1:5000 in AB media (without trace elements and vitamins). Twenty microliters of test samples were mixed with 180 µl of diluted cell suspension in white microtitre plates (NUNC, Roskilde, Denmark) and incubated at 30°C with orbital shaking (650 rpm). Luminescence was measured hourly using a Victor Wallac luminescence reader. Chemically synthesized DPD (Omm Scientific Inc., TX) or AB media were used as positive or negative controls, respectively.

The results presented as fold induction were obtained by dividing the luminescence of the samples with sterile AB medium for each time point. The maximum activity of fold change was referred to as AI-2 activity.

7.2 Standard RNA techniques

7.2.1 Sample preparation for RNA isolation from *S. mutans*

7.2.1.1 Preparing planktonic cells for RNA extraction

Media containing planktonic grown cells was mixed with the double volume of RNA protect (Qiagen), incubated for at least 5 minutes at RT and centrifuged to remove the supernatant. Pellets were frozen at -70°C until RNA extraction.

7.2.1.2 Preparing biofilm grown cells for RNA extraction

The formation of biofilm was routinely carried out in 96 well microtitre plates. The supernatant was removed by pipeting and the biofilm was carefully washed two times with PBS. Afterwards the biofilm of 10 wells was covered with RNA protect, incubated for 5 minutes at RT and scraped from the bottom using a spatula. The cell suspension from all wells was pooled, pelleted and the pellet was stored at -70°C until RNA extraction.

7.2.1.3 Preparing flow cytometric separated cells for RNA extraction

Cells were separated according to their GFP intensity, using the FACSAria™ II Cell Sorter System (Becton Dickinson) and PBS as sheath fluid, and directly collected in RNA Protect. The cell suspension was filtered through a 0.2 µm filter. The filter was stored at -70°C until RNA extraction.

7.2.2 RNA isolation

Pellets or filters containing cells were resuspended or overlaid with 2.5 mg lysozyme and 50 U mutanolysin, solved in TE Buffer (200µl for cell pellets or 1000 µl for filters, pH = 8.0) and incubated for 25-45 minutes. RLT buffer (Qiagen) containing sterile, acid washed glass beads (diameter 106 µm) was added and vortexed for 3 min. Subsequent RNA extraction was carried out using the RNeasy micro/mini kit (Qiagen) according to the manufacturer's instructions. For extraction of RNA from biofilms, two additional washing steps were performed, using 80% (v/v) ethanol, prior elution.
Genomic DNA was removed using the DNAse I (Qiagen) on column digestion protocol. The quality of the total RNA was controlled on a denaturating formaldehyde agarose gel and the absence of gDNA with a standard PCR.

7.2.3 Denaturating gel electrophoresis for RNA

For RNA gel electrophoresis, 0.6 g agarose was dissolved in 5 ml 10x FA buffer and 45 ml autoclaved Milli-Q water by heating in a microwave. Towards cooling down, 0.9 ml 37% formaldehyde and 1 µl ethidium bromide (10 mg/ml) were added and the gel was casted. RNA samples were mixed with 5x loading buffer, incubated at 65°C for 5 minutes and put on ice prior loading onto the gel. Electrophoresis was started with 50 V until the samples entered the gel matrix and continued at 70 V until visualization using UV light.

10x FA gel buffer
200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)
50 mM sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

7.2.4 Microarray

7.2.4.1 Design of the microarray

Using the earray-platform from Agilent (https://earray.chem.agilent.com/earray/) a customized whole genome microarray of *S. mutans* UA159, for total RNA as specimen, was designed. The array contained all open reading frames (ORF) and non coding regions of *S. mutans*. Each ORF and intergenic region was covered with three antisense probes (60 bp in length) in duplicate. Positive and negative controls from Agilent were included in the design.

7.2.4.2 RNA labeling

Labeling of RNA was carried out with the ULS labeling kit (Kreatech, Germany), using Cy3/Cy5 as fluorescence dyes, according to the manufacturer's instructions.
The reaction mixture contained 2 µl of 10x labeling solution, 1 µl of Cy-ULS dye and 2 µg of total RNA (minimum concentration of 117 ng/µl). The reaction was filled up with RNase free water to 20 µl. Reference RNA was labelled using Cy3 and sample RNA was labelled using Cy5. The reaction was incubated for 30 minutes at 85°C and kept on ice until removal of unbound dye.

7.2.4.3 Dye removal of labeled RNA

1. Re-suspend column material by vortexing
2. Loosen cap ¼ turn and snap off the bottom closure
3. Place the column in a 2 ml collection tube
4. Pre-centrifuge the column for 1 minute at 20,800 x g
5. Discard the flow-through and column cap
6. Add 300 µl of water to the column and centrifuge for 1 min at 20,800 x g
7. Discard flow-through and collection tube
8. Place column into a 1.5 ml collection tube
9. Add labelling solution onto the centre of the column bed
10. Centrifuge for 1 min at 20,800 x g

11. Flow-through contains the purified labelled DNA

7.2.4.4 Determination of the degree of labeling (DoL)

To ensure that the RNA was properly labelled and could be used for microarray hybridisation, the DoL was determined. Therefore, 2 µl of labelled RNA was measured with the NanoDrop (Thermofischer Scientific) (program: microarray). The degree of labelling was calculated using a web form on the manufacturer's homepage. (http://www.kreatech.com/Portals/kreatech/downloads/labeling/27_DoL%20calculator_28082007.xls)

7.2.4.5 RNA fragmentation

For the fragmentation 450 ng of each RNA population (Cy3 and Cy5 labelled RNA) was mixed, 5 µl of 10x blocking agent (Agilent) was added and the reaction was filled up to 25 µl with water. The reaction was incubated for 30 min at 60°C until 25 µl of 2x GE hybridisation buffer (Agilent) was added. The reaction was stored on ice until hybridisation.

7.2.4.6 RNA hybridisation and scanning

Hybridisation was carried out at 65°C for 17 hours using the Agilent hybridisation chamber. Scanning was achieved using the Agilent DNA microarray scanner.

7.2.4.7 Data analysis

Background correction was performed using the Agilent scanner algorithms and raw data were extracted using the agilent feature extraction software. Data processing was carried out with the Bioconducter – Linear Models for Microarray analysis (LIMMA) package (Wettenhall and Smyth 2004) using the R language (http://www.r-project.org). Genes or non-coding regions with a P-value < 0.05 were selected for further analysis.

7.2.5 Reverse transcription

Synthesis of cDNA was carried out using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) or using the Quantitect reverse transcription kit (Qiagen) according to the manufacturer's instructions.

Protocol for the Quantitect reverse transcription kit

Step 1

RNA	up to 1 µg
gDNA wipeout	2 µl
water	ad 14 µl

Incubate for 2 min at 42°C, place on ice and add:

Step 2

Primer mix	1 µl
5x buffer	4 µ
RNA mix from step 1	14 µl
RT enzyme	1 µl

Incubate at 42°C for 15-30 minutes, heat to 95°C for 3 min.

7.3 Standard DNA techniques

All reactions were performed in 0.2 ml PCR soft tubes in an Eppendorf thermocycler. The DNA was separated routinely on 1% (w/v) TAE agarose gels (NEED ultra-quality agarose, Roth). For DNA smaller than 500 bp, 2% (w/v) agarose was used. DNA was visualized by staining in an ethidium bromide (2 µg/ml) bath for 15-30 min.

7.3.1 PCR

7.3.1.1 Standard PCR

All standard PCRs were routinely carried out using Taq polymerase from Qiagen. Primer were dissolved in water to a concentration of 10 µM. The reaction mixture was assembled according to the manufacturer's instruction, containing 3 mM $MgCl_2$, 200 µM of dNTPs, 200 nM of each primer and 0.25 U/µl Taq polymerase.
The program consists of a 5 min pre-denaturating step at 94°C, followed by 30-35 cycles of denaturation for 10 sec at 94°C, annealing for 25 sec at 50-60°C and elongation for x min at 72°C. The program was finished by a 10 min post elongation step at 72°C.

7.3.1.2 High fidelity PCR

High fidelity PCRs were carried out using Pfu polymerase (Promega). All conditions were similar to the standard PCR, except that $MgCl_2$ was replaced by $MgSO_4$ and that 0.025 U/µl Pfu polymerase was used.

7.3.1.3 Inverse PCR

Inverse PCR was used to delete parts of or for mutation of plasmids, respectively. Therefore the Crimson LongAmp® Taq polymerase (NEB) was used following the manufacturer's protocol. Reactions were carried out in a volume of 50 µl and additionally 0.75 U of Pfu polymerase was added. Afterwards the reaction mixture was treated with DpnI, purified, blunted, re-ligated and transformed into *E. coli,* to obtain the modified plasmid.

7.3.1.4 Quantitative RT- PCR

Quantitative RT-PCR was performed using the LightCycler 480 system (Roche, Mannheim, Germany) and the reaction mixtures were prepared using the Quantitect SYBR Green PCR Kit (Qiagen). Changes in the level of gene expression were calculated automatically by the LightCylcer 480 software using the $\Delta\Delta C_T$ method. The gyrase A gene (SMU.1114) was used as the housekeeping reference gene. For each reaction, 5 µl primer mix (1 µM of each primer), 5 µl of diluted cDNA and 10 µl of the Quantitect SYBR Green Solution was mixed and transferred into a well of a 96 well LightCycler standard plate.

All subsequent PCR steps were performed according to the manufacturer's protocols. All measurements were done in duplicate.

7.3.2 Purification of DNA

For removal of nucleotides, proteins, salts, etc., the PCR purification kit from Qiagen was used. For extraction of single bands from agarose gels, the gel extraction kit (Qiagen) was used. To concentrate DNA preparations, the DNA was precipitated by adding 1/10 volume 3 M sodium acetate and 2.5 volume of 96% (v/v) ethanol. The mixture was incubated overnight at –70°C. Afterwards, the mixture was centrifuged at 14,000 rpm in an Eppendorf table top centrifuge and washed with 80% (v/v) ethanol. This procedure was repeated twice. The DNA pellet was air dried and resuspended in the appropriate volume of buffer.

7.3.3 Restriction digestion

Restriction enzymes were purchased from New England Biolab (NEB) or Fermentas but reactions were carried out using the buffers from NEB. For analytical purposes, reactions were carried out in a volume of 20 µl using routinely 500-1000 ng DNA and 1-5 units of restriction enzyme.
For preparative digestions the volume was scaled up to 50 µl and up to 5 µg of DNA was used. Reactions were carried out at 37°C (25°C in the case of SmaI) for 2 hours, followed by heat inactivation at 65/80°C for 20 minutes.

7.3.4 Generation of blunt ends

For generation of blunt ends, T4 DNA polymerase from Fermentas was used. The reaction was performed as recommended in a volume of 20 µl using up to 1 µg of DNA and 1 Unit of enzyme. The mixture was incubated at 11°C for 20 minutes and heat inactivated at 75°C for 10 minutes. DNA was not further purified and directly used for downstream applications.

7.3.5 Dephosphorylation of DNA

Before the ligation of inserts, digested plasmids were dephosporylated using shrimp alkaline phosphatase (SAP, Fermentas). A suitable amount of SAP was added directly to the heat inactivated restriction digestion reaction and incubated at 37°C for 30 minutes.

7.3.6 Ligation

Routinely, ligation reactions were performed in a volume of 20 µl, using 1 weiss unit of T4-Ligase (Fermentas) and were incubated overnight at 16°C. For the ligation of inserts into plasmids, 50-100 ng of plasmid and a three molar excess of insert was used. For facilitating blunt end ligations 10% (w/v) PEG 4000 was added.
To perform restriction and ligation simultaneously, the reaction was carried out in the designated restriction enzyme buffer, supplemented with 1 mM ATP and 10% (w/v) PEG 4000. Restriction enzyme and ligase were added to a concentration of 1 (weiss) Unit. The mixture was incubated at 25°C for 3 hours, followed by overnight incubation at 4°C. Ligation reactions that contained no PEG were heat inactivated at 65°C for 15 minutes prior transformation.

7.3.7 Isolation of DNA

7.3.7.1 Isolation of genomic DNA from *Streptococcus mutans*

Two millilitres of an overnight culture was pelleted and resuspended in 200 µl LM buffer and incubated at 37°C for 45 minutes. Afterwards 200 µl of buffer T1 from the Nucleospin Tissue kit (Macherey & Nagel) was added and incubated at 70°C for 10 minutes. Subsequent steps were performed using the Nucleospin Tissue kit according to the manufacturer's instructions.

7.3.7.1 Isolation of plasmid DNA from *Escherichia coli*

For analytical or preparative purposes the fast plasmid isolation kit (Eppendorf) or the plasmid isolation kit from Macherey & Nagel was used according to the manufacturer's instructions, respectively.

7.3.8 Transformation

7.3.8.1 TSS-transformation of DNA into *E. coli*

An overnight culture was diluted 1:100 in 10 ml of fresh LB media and incubated for two hours. Subsequent 1 ml was shortly pelleted, the supernatant discarded and the pellet resuspended in 100 µl ice-cold TSS buffer. Up to 10% (v/v) of DNA was added to the cells and gently mixed. The mixture was put on ice for 30 minutes, followed by 30 sec incubation at 42°C in an water bath. Afterwards cells were chilled on ice for 1 min and 250 µl SOC media was added. Cells were incubated at 37°C for one hour, prior plating on selective agar plates.

7.3.8.2 DNA transformation into *S. mutans*

For routine transformation, an overnight culture grown in THBY was diluted 1:10 in fresh media and incubated at 37°c at aerobic conditions. Afterwards 0.2 µM CSP and DNA were added and the mixture was further incubated for three hours. Before plating on selective agar plates the cells were briefly sonicated (20x 0.5 sec pulse, 0.5 sec break, MS72 probe with 10% power; Bandelin Sonoplus HD2200, Berlin, Germany).

7.3.8.3 Competence assay for *S. mutans*

Overnight cultures were diluted 1:20 in fresh media and incubated until an OD ~ 0.6 had been reached. Afterwards cultures were diluted 1:10 in fresh media and further incubated until an OD of 0.2-0.25 had been reached. Synthetic CSP was added to a concentration of 0.4 µM and cultures were incubated for 20 min, until 1µg/ml (final concentration) plasmid DNA was added. After two hours of incubation serial dilutions and plating on selective and non-selective media were carried out. The transformation efficiency was calculated by dividing the number of transformants by the total cell number. For comparing the transformation

capacities of WT and the *spxB* mutant, plasmid pLSE4 (Diaz and Garcia 1990) was used. For all other purposes plasmid pVA-GTFA (Li et al. 2001b) was.

7.3.9 Construction of knockout mutants in *S. mutans*

For the construction of knockout mutans the PCR ligations mutagenesis method according to Lau *et al*. was used (Lau et al. 2002).
Therefore, approximately 1 kb upstream (primer P1/P2) and downstream (primer P3/P4), containing 50-200 nucleotides of the begin and end of the coding sequence, were amplified using PFU polymerase. Primer P2 contained a restriction site for AscI and primer P3 had a restriction site for FseI. An erythromycin cassette was amplified from plasmid pALN122 containing either AscI or FseI at both ends, respectively. All fragments were digested with the appropriate enzymes and subsequent ligated. The ligation mixture was directly transformed into *S. mutans*. Erythromycin resistant clones were confirmed by PCR and sequencing.

7.4 Biofilms

7.4.1 Biofilm formation

For biofilm formation, overnight cultures were diluted 1:100 in the designated media and grown for 4 hours. Prior start of biofilm formation, 1% (w/v) sucrose was added to the cultures. Twenty microliters of different compounds (e.g. AI-2) or water was pipetted into the wells of a 96 well microtitre plate and mixed with 180 µl of the cell suspension. Plates were carefully sealed with parafilm and incubated for the stated time.
For biomass quantification or CLSM analysis, polystyrene (TPP, Switzerland) or plates with glass bottom (Greiner, Germany) were used, respectively

7.4.2 Biomass determination using crystal violet

At first, the optical density was determined at 620 nm using a Wallac Victor3 1420 Multilabel Counter, Perkin-Elmer Life Sciences. Afterwards unattached bacteria and media were removed by decanting the plate. The remaining loosly bound cells were removed by adding

200 µl PBS to each well . The PBS was removed by decanting and blotting on paper towels. The washing procedure was repeated twice. Plates were allowed to air dry before addition of 200 µl of 0.1% crystal violet (w/v) for 15 min at room temperature. The wells were washed with 200 µl PBS, as described above, until the washing solution was transparent. The bound dye was extracted by adding 200 µl ethanol (96%) and orbital shaking for 2h. One hundred microliters were transferred to a new plate and biofilm formation was quantified at 620 nm using an Wallac Victor3 1420 Multilabel Counter (Perkin-Elmer Life Sciences).

7.4.3 Biofilm structure determination using CLSM

Unattached and loosly bound bacteria were removed as described above. Afterwards, 100 µl of the LIVE/DEAD *Bac*Light bacterial viability staining kit L13152 (Invitrogen, Molecular Probes, 20 Inc. Eugene, OR, USA) was added and incubated for 15 minutes in the dark. Biofilms were observed using an Olympus FluoView 1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). For visualization of fluorescence an Ar-laser (488 nm), and He-laser (561 nm) and the appropriate emission filter sets (Syto9: 500-545 nm; PI: 580-680 nm) were used. Image data were subsequently processed using the Imaris software (version X64 5.7.2, Bitplane AG, Zürich, Switzerland).

7.5 Single cell analysis

7.5.1 Fluorescence microscopy

Cells were pelleted, washed and resuspended using PBS. For chain disruption, cells were sonicated (see 7.3.8.2). For imaging an Olympus BX60 microscope, equipped with a colorview II camera and a 100/1.3 oil immersion objective was used. The filter U-MWIBA3 (excitation 460-495 nm, emission 510-550 nm, dichromatic filter 505 nm) from Olympus (Seelze, Germany) was used to visualize eGFP with an exposure time of 5 sec. The filter 20HE (excitation 546 ± 12nm, emission 607 ± 80 nm, dichromatic filter 560 nm) from Carl-Zeiss (Zaventum, Belgium) was used to visualize Cy3 with an exposure time of 10 sec. The filter U-MWIY2 (excitation 545-580 nm, emission 610IF, dichromatic filter 600 nm) from Olympus was used to visualize PI with an exposure time of 1 sec. Images were recorded under the same condition, using the cellB software from Olympus. For better visualizations on

print-outs, brightness and contrast were modified equally for all images using Adobe Photoshop.

7.5.2 Visualization of DNA uptake

Overnight cultures were 1:10 diluted in fresh media and incubated as described above. CSP was added after 1h to an end concentration of 0.2 µM and cultures were incubated further for 2 hours. 100 µl of cells were withdrawn and a ~ 2 kb DNA fragment (PCR amplified), labelled with Cy3 (ULS labelling, see above), was added at a final concentration of 1µg/ml and incubated for 15 min at 37°C. Afterwards, 30 Units of DNase I (Roche, Mannheim, Germany) were added and incubated for 5 min at 37°C. Cells were washed, resuspended in PBS and visualized using fluorescence microscopy.

7.5.3 Flow cytometry

7.5.3.1 Cell sorting of strain SMGFPcomX

All media used for flow cytometry were filtered through a 0.22 µm filter. For subpopulation sorting an overnight culture of *S. mutans* SMGFPcomX was diluted 1:10 into fresh medium and incubated at 37°C. After one hour, induction with 0.2 µM CSP was carried out. Cells were further incubated for two hours and sonicated as described for disrupting cell chains for fluorescence microscopy prior to flow cytometry. This procedure was repeated every half hour and every sample was subjected to sorting for 30 minutes. In total 10 individual samples were sorted, yielding a single biological sample. Control cells of a mixed induced and not induced culture were sonicated and kept in RNA protect until the sorting was finished. Flow cytometric sorting was performed on a FACSAria™ II Cell Sorter System (Becton Dickinson). Filtered PBS was used as the sheath fluid. Cells were kept at 37°C during sorting and were collected directly in RNA protect. Forward and side scatter were set to log with a threshold of 200 on both parameters. Detection of GFP fluorescence was through a 525/50 nm bandpass filter. PMT Voltage was 370 for FSC (forward scatter), 205 for SSC (side scatter), and 467 for GFP. Despite sonication, chains of two or more cells still occurred in the culture. We used pulse-width (-W) and –area (-A) of FSC and SSC to identify these doublets and chains. When a cell/particle passes through the laser beam, it will generate a signal pulse (signal over time), which has a height (-H), width (-W) and integrated area (-A). Single cells

of different sizes will show a constant pulse width and strongly correlated height to area ratio. Doublets and other aggregates will show a similar pulse height, but can be identified by their increased pulse width and area. According to the FSC-W and SSC-W plots, gates were set more stringent to allow sorting of single cells. Not induced cells reached a fluorescence intensity of approx. 800 (arbitrary units), thus cells with a higher value were sorted as GFPplus and cells with a lower value as GFPminus. Before cells were collected in RNA protect, the efficiency of sorting was determined and both subpopulations were subjected again for analysis. The whole experiment was done in duplicate and each replicate was used separately for transcriptome analysis.

7.5.3.2 Analysis of GFP intensity

To measure the GFP intensity of all reporter strains, cells were induced and sonicated as described above (see 7.3.8.2) and 100.000 cells were analysed. The mean values of GFP positive cells (intensity > 800 units) from two biological replicates were determined.

7.6 Reporter gene assays

7.6.1 Luciferase measurement

Luciferase assays were performed by withdrawing 1 ml culture. The OD_{600} was measured and samples were held on ice until the start of the assay. 100 µl of each sample were mixed with 3x assay buffer (75 mM tricine, 15 mM $MgSO_4$, 1.5 mM EDTA, 1.5 mM DTT, 900 µM ATP, 3 mg/ml (w/v) BSA, and 3% (w/v) D-Glucose, pH = 7.8) and incubated 10 min prior to injection of 100 µl D-luciferin (120 µM final concentration) solved in 20 mM tricine (pH 7.8). D-luciferin (Carl-Roth, Karlsruhe, Germany) was resuspended in 20 mM tricine (pH = 7.8, 1 mg/ml), aliquoted and stored at –70°C until use. Luminescence was recorded for 35 s (POLARstar OPTIMA luminometer, BMG LABTECH; or on an victor Wallac luminescence reader, Perkin Elmer) and normalized against the OD_{600} to calculate the relative light units (RLU). For calculation of the fold change, the RLU were normalized against the RLU of time zero. All measurements were done in triplicate.

7.6.2 Fluorescence measurement

Cells were pelleted, washed, resuspended using PBS and transferred into a 96 well microtitre plate. Fluorescence was measured in a microtitre plate reader (Wallac Victor3 1420 Multilabel Counter, Perkin-Elmer Life Sciences) equipped with filters suitable for excitation (488nm) and emission (535 nm) of GFP.

7.7 Protein techniques

7.7.1 SDS-PAGE

SDS-PAGE was carried out according to standard procedures using a biometra gel electrophoresis system (Laemmli, 1970, Sambrook & Gething, 1989). Proteins were separated using a 15% running gel that was overlayed with a 4% stacking gel. Protein samples were mixed with loading buffer and boiled at 100°C for 5 minutes. The samples were briefly centrifuged and loaded onto the gel. Electrophoresis was carried out in SDS running buffer at 10V/cm at ambient temperature and the gel was stained using the PageBlue protein staining solution (Coomassie) from Fermentas.

Running gel

Water	3.6 ml
1.5 M Tris/HCl pH 8.8	3.8 ml
Acrylamide/bisacrylamide (37.5 : 1)	7.8 ml
10% (w/v) SDS	150 µl
TEMED	20 µl
25% (w/v) APS	20 µl

Stacking gel

Water	2.9 ml
0.5 M Tris/HCl pH 6.8	1.26 ml
Acrylamide/bisacrylamide (37.5 : 1)	0.85 ml
10% (w/v) SDS	50 µl
TEMED	10 µl
25% (w/v) APS	15 µl

Loading buffer

Tris base	1.52 g
Glycerol	20 g
SDS	4.6 g
Bromphenolblue	0.05 g
Beta-mercaptoethanol	10 ml
Water	ad 0.05 L

Adjust pH with HCl to pH 6.8

SDS running buffer

Glycine	14.4 g
Tris base	3.03 g
SDS	1 g
Water	ad 1 L

7.7.2 Western Blot

For detection of 6His-MleR, proteins from the SDS-PAGE were blotted onto a PVDF membrane using a semi-dry blotting system from NOVEX. The membrane was equilibrated with methanol. The whatman papers (Schleicher & Schuell, Germany) and the transfer chamber were equilibrated with blotting buffer.
Afterwards, two whatman papers (3 mm), the gel, the membrane and two whatman papers (3 mm) were assembled within the transfer chamber. Blotting was carried out for one hour at 120 mV. After transfer the membrane was washed two times for 10 min in 20 ml 1x TBS buffer. Afterwards the membrane was incubated for 20 minutes in 20 ml blocking solution and washed two times for 10 min in 20 ml 1x TBSTT and again 10 min with 20 ml 1x TBS. The membrane was incubated overnight in 20 ml blocking solution containing the 1:1000 diluted anti-his-tag monoclonal antibody (Novagen). The membrane was washed twice for 10 minutes with 20 ml 1x TBSTT buffer and once again with 20 ml 1x TBS for 10 minutes. The membrane was incubated for 1 h with the secondary goat anti-mouse antibody which was diluted 1:5000 in 8 ml blocking solution. The membrane was washed five times for 10 minutes with 20 ml 1x TBSTT and drained afterwards. The membrane was covered with CDP-Star AP substrate (Novagen) and afterwards visualised using a phosphoimager (Biorad).

Blotting buffer

Tris base	25 mM

Glycine	192 mM
Methanol	20% (v/v)

1x TBS

20x TBS (Novagen)	dilute 1:20 in water

1x TBSTT

20x TBSTT (Novagen)	dilute 1:20 in water

Blocking solution

5% (w/v) alkali soluble casein (Novagen)	dilute 1:5 in water

7.7.3 Expression and purification of MleR

For expression, *E. coli* Tuner carrying pALEC30 was grown in an 250 ml LB culture to an OD_{600nm} of 0.6 and expression was induced by adding IPTG to a final concentration of 1 mM. The cells were grown for additional two hours, harvested (4,000 x *g*, 20 min, 4°C) and resuspended in 50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 25 mM imidazole and 5 mg/ml lysozyme and incubated on ice for 30 min. Subsequently, the cells were further lysed by sonification (4x 1 min pulse, 1 min break, MS72 probe with 25% power; Bandelin Sonoplus HD2200, Berlin, Germany) and the soluble 6His-MleR extract was separated from insoluble cell material by centrifugation (25,000 x $g_{, 30 \text{ min}, 4°C)}$. The 6His-MleR protein was then purified by IMAC affinity chromatography using Talon resin (Clontech, Saint-Germain-en-Laye, France). Bound protein was washed with 8 bed volumes 50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 25 mM imidazole and eluted with 50 mM NaH_2PO_4, pH 7.0, 300 mM NaCl, 300 mM imidazole. The eluted 6His-MleR protein (purity >90% on an SDS-PAGE) was always stored on ice and was verified by western blot (Anti His-tag antibody, Novagen) and N-terminal sequencing.

7.7.4 EMSA

For binding studies, the purified MleR protein was dialysed four times against 1 liter 1x binding buffer (20 mM Tris, pH 7.5, 100 mM KCl, 2 mM EDTA, 10% glycerol) at 4°C for 12 hours using a 12-14 kDa cut-off dialysis bag (Medicell International Ltd., London, UK).

Several fragments of the region between *mleR* and *mleS* were PCR amplified and directly used for gel retardation experiments (see Table 8 for primers). To verify the specificity of the DNA-MleR interaction each reaction mixture contained an equal amount of competitor DNA. Competitor DNA consisted either of an internal fragment of *mleS*, amplified by PCR (primers 137qF/R), or a DNA fragment within the upstream region of *mleR*, generated by hybridising complementary primers (EP10/11, Table 8). For this purpose, primers EP10/11 were mixed in equal molar ratios, denaturated by heating to 100°C and annealed by slowly cooling down to room temperature. DNA fragments, MleR protein (appr. 100 ng) and competitor DNA (in case of the complementary primers 75 ng/µl, final concentration) were mixed and incubated for 20 min at ambient temperature. To further exclude unspecific interactions, MleR was substituted with 100 ng BSA (Carl-Roth) and tested for each fragment. The reaction mixtures were subsequently loaded onto a 0.5x TBE, 4.5% polyacrylamide (37.5:1, acrylamide/bisacrylamide) gel. Since the MleR protein has a calculated pI of ~9, DNA in complex with MleR was hardly entering the gel using pH values below 9.2. Therefore the pH of the gel cast solution and electrophoresis buffer were adjusted to pH 9.45. L-malate was added to the binding reaction, the gel and the electrophoresis buffer (0.5x TBE) at 5 mM final concentration when needed. Electrophoresis was carried out at 10V/cm at ambient temperature and the gel was stained using SYBR Gold (Invitrogen).

7.8 Miscellaneous assays

7.8.1 Acid and hydroxide peroxide killing

Overnight cultures of *S. mutans* strains were diluted 1:20 in fresh THBY medium (pH 7) and grown under aerobic conditions. Cultures were harvested (at an OD_{600} ~ 0.3) by centrifugation at 11,000 x *g* for 5 min. The supernatant was carefully discarded and the pellet was resuspended in 0.1 M glycine buffer pH 7.0 (time zero) or pH 3.1 without malate (control) or in the presence of 25 mM L-malate. Samples of cells incubated at pH 3.1 were withdrawn after 20, 40, 60, and 80 minutes, serially diluted in 0.1 M glycine buffer, pH 7.0, and plated on THBY plates in triplicate and incubated for 48h aerobically.
For pre-induction of the acid tolerance response and to achieve maximal expression of MLF, cells were grown in THBY (pH 5.5) in the presence of 25 mM L-malate and treated as described above. To determine the capability to withstand hydrogen peroxide, cells were collected as described above and resuspended in 0.1 M glycine buffer, pH 7.0. Before the addition of H_2O_2, 0.2% (v/v) final concentration, an aliquot was withdrawn to determine the cell number by colony forming units at time zero. To inactivate hydrogen peroxide, catalase

(5 mg/ml, Sigma) was added immediately after sampling. Samples were serially diluted in 0.1 M glycine buffer, pH 7.0, plated in triplicate and incubated as described above.

7.8.2 Assay for malolactic fermentation activity

The capacity to carry out malolactic fermentation was determined by the method of Sheng and Marquis (Sheng and Marquis 2007), slightly modified. Briefly, *S. mutans* cells were cultivated in THBY aerobically until the end of the log phase. An equal amount of wildtype and *ΔmleR* cells was harvested by centrifugation (5000 x *g*, 15 min, 4°C) washed with salt solution (50 mM KCl + 1 mM $MgCl_2$) and incubated for 1h in 20 mM potassium phosphate buffer, pH 7.0 at 37°C. The pH of the cultures was adjusted with HCl to pH 6.3. Prewarmed L-malate was added to the cell suspension (42 mM end concentration) to initiate malolactic fermentation. Aliquots were withdrawn after 0, 20, 40, and 60 minutes and 12 hours for measuring the pH and the L-malate concentration of the supernatant using the L-malic acid kit from Biosentec (Toulouse, France). For determination of L-malate in growing cultures, 1 ml was centrifuged at 11,000 x *g* for 5 min and the supernatant was analysed using the L-malic acid kit.

7.8.3 Assay for the production of mutacin IV and V

Overnight cultures of *S. mutans* were washed three times in fresh media, without antibiotics, and 20 µl were spotted on THBY agar plates and dried for 20 minutes. Afterwards, 30 µl of the overnight culture of the indicator strain was resuspended in 3 ml THBY containing 1% (w/v) agar, poured into the petri dish and dispersed over the whole surface. To determine the production of mutacin IV, *Streptococcus sanguis*, and for mutacin V, *Lactococcus lactis* 4067, were used.

7.9 Construction of plasmids

7.9.1 Construction of the luciferase reporter plamids

For the construction of the luciferase reporter strains, the advanced firefly luciferase was amplified using *Pfu* polymerase from plasmid pHL222 using primers lucF/lucR. The amplicon was cloned into the suicide vector pFW5 (Podbielski et al. 1996) via the NcoI and SpeI sites to generate plasmid pALEC15. Afterwards the upstream region, containing the promoter of interest was amplified using PFU polymerase and incorporated within the NcoI restriction site. The reporter plasmid was integrated into the genome of *S. mutans* by single homologous recombination and luciferase activity was measured as described above.

7.9.1 Construction of the GFP reporter strains

For the construction of the GFP fluorescence reporter plasmids, a fragment containing the *recA* promoter from *S. pyogenes* and GFP was released from pVA-EGFP2 (Nakagawa et al. 2001) by XbaI and religated, yielding pALSM01. The eGFP from pKRC12 was amplified using primer GFPF/R, cut with XbaI/NheI and ligated to pALSM01, giving pALSM02. Afterwards the vector was digested with PciI and EcoRI, blunted and religated to release the *lac* promoter, giving pALSM03. Subsequently, the upstream regions of *comX* (PcomX2F/R), *cipB* (PcipBF/R) and *comE* (PcomE2F/R) were amplified and cloned into the EcoRV site. The plasmids were confirmed by sequencing and transformed into the wildtype of UA159.

7.9.2 Construction of the MleR overexpression plasmid

For expression the coding sequence of *mleR* was amplified using primers CDSMleRF/R and cloned into the pET28c expression vector (Novagen, Merck KgaA, Darmstadt, Germany) via the NdeI and NheI restriction sites. The resulting plasmid pALEC30 was sequenced for confirmation.

7.9.3 Construction of the *sahH* integration plasmid

For heterologous expression of the *sahH* gene, the plasmid pFW5-SahH for allelic replacement of the *erm* cassette of the Δ*luxS* strain was constructed. Therefore, the *sahH* gene was amplified from *Acinetobacter baylyi* ADP1 using primers SahHFor/Rev. Afterwards the constitutive promoter of the spectinomycin cassette (*aad9*) was amplified (PaadFor/Rev) using the pFW5 plasmid as template and cloned in front of the *sahH* gene. Finally, upstream

(UpluxSF/R) and downstream (DoluxSF/R) fragments of the *luxS* locus were cloned flanking the *sahH-aad9* fragment. The plasmid was transformed into the *luxS* mutant of *S. mutans*. Spectinomycin resistant colonies were confirmed by PCR and sequencing. To verify transcription, RT-PCR for the full length *sahH* gene was carried out (not shown).

7.9.4 Construction of the *luxS* complementation plasmid

For *in-trans* complementation, the coding sequence of the *luxS* gene and 500 bp upstream were amplified using primers PluxSF/CDSluxSR and cloned into the shuttle vector pDL278, in orientation to the *lacZ* promoter, to generate pDLluxS.

7.9.5 Construction of the *spxB* overexpression plasmid

For overexpression, the coding sequence of *spxB* including an artificial ribosomal binding site was amplified with primers spxBCDSF/R and cloned into the SmaI site of vector pIB166 (Biswas et al. 2008b) giving plasmid pIBspxB. Both plasmids were transformed into SMluccomX to monitor the luciferase activity.

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8 Appendix

8.1 Supplementary table 1

Supplementary table S1.

Genes that were significant changed compared to the wildtype. Significant changes (fold change > ±1.7; P-value < 0.05) are marked in bold.

Δ*luxS*$_2$ **up-regulated genes: 20** **down-regulated genes: 17**				
Locus tag	**description**	**Gene symbol**	**Fold Change**	**P-value**
ABC transporters (4)				
SMU.933	putative amino acid ABC transporter, periplasmic amino acid-binding protein	-	-2,66	2,5E-04
SMU.934	putative amino acid ABC transporter, permease protein	-	-2,68	5,8E-04
SMU.935	putative amino acid ABC transporter, permease protein	-	-2,69	4,6E-04
SMU.936	putative amino acid ABC transporter, ATP-binding protein	-	-2,64	4,7E-04
Alanine, aspartate and glutamate metabolism (1)				
SMU.366	glutamate synthase subunit beta	gltD	1,74	3,2E-04
Arginine and proline metabolism (4)				
SMU.663	N-acetyl-gamma-glutamyl-phosphate reductase	argC	1,88	2,1E-03
SMU.665	acetylglutamate kinase	argB	2,00	5,9E-04
SMU.664	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	argJ	1,74	2,1E-03
SMU.666	acetylornithine aminotransferase	argD	1,75	2,5E-02
Citrate cycle (TCA cycle) (3)				
SMU.670	aconitate hydratase	citB	2,03	1,0E-04
SMU.671	citrate synthase	citZ	2,94	4,4E-05
SMU.672	isocitrate dehydrogenase	idh	3,00	2,3E-05
Cysteine and methionine metabolism (1)				
SMU.474	S-ribosylhomocysteinase	luxS	-83,31	1,8E-04
Energy metabolism (1)				
SMU.962	putative dehydrogenase	-	-2,11	7,4E-04
Glutathione metabolism (1)				
SMU.1296	putative glutathione S-transferase YghU		-1,80	4,4E-06
Glycolysis / Gluconeogenesis (3)				
SMU.1421	branched-chain alpha-keto acid dehydrogenase subunit E2	pdhC	1,86	8,5E-03
SMU.1422	putative pyruvate dehydrogenase E1 component beta subunit)	pdhB	2,12	3,9E-02
SMU.1424	putative dihydrolipoamide dehydrogenase	pdhD	1,77	4,1E-04
Hypothetical proteins (10)				
SMU.1818c	hypothetical protein	-	1,72	2,3E-02

SMU.1373c	hypothetical protein	-	1,73	8,0E-04
SMU.63c	hypothetical protein	-	-1,75	3,2E-06
SMU.175	hypothetical protein	-	1,97	1,7E-02
SMU.399	hypothetical protein	-	-1,88	1,2E-08
SMU.673	hypothetical protein	-	2,18	4,4E-05
SMU.753	hypothetical protein	-	2,61	2,9E-08
SMU.932	hypothetical protein	-	-2,96	2,5E-04
SMU.941c	hypothetical protein	-	-1,83	4,0E-02
SMU.961	hypothetical protein	-	-1,99	8,2E-04
Miscellaneous (2)				
SMU.400	putative secreted esterase	-	-1,76	1,6E-06
SMU.940c	putative hemolysin III	-	-1,71	2,7E-02
Phenylalanine, tyrosine and tryptophan biosynthesis (2)				
SMU.1836	phospho-2-dehydro-3-deoxyheptonate aldolase	aroG	-2,40	1,6E-03
SMU.1837	phospho-2-dehydro-3-deoxyheptonate aldolase	aroH	-24,59	5,6E-04
Transcription (3)				
SMU.1246c	putative transcriptional regulator	-	1,75	3,3E-05
SMU.1657c	putative nitrogen regulatory protein PII	-	2,33	3,1E-05
SMU.2084c	transcriptional regulator Spx	spxB	2,19	3,7E-08
Transporter (2)				
SMU.1658	putative ammonium transporter, NrgA protein	nrgA	2,15	1,5E-04
SMU.2059c	putative integral membrane protein	-	-1,73	2,1E-05

8.2 Supplementary table 2

Supplementary table S2.

Genes that were significant changed compared to the wildtype. Significant changes (fold change > ±1.7; P-value < 0.05) are marked in bold.

Δ*luxS*$_2$pDLluxS **up-regulated genes: 147** **down-regulated genes: 193**				
Locus tag	**Description**	**Gene symbol**	**Fold Change**	**P-value**
ABC transporters (24)				
SMU.370	putative ABC transporter, ATP-binding protein	-	-1,84	3,1E-07
SMU.431	putative ABC transporter, ATP-binding protein	-	2,08	2,8E-05
SMU.432	putative ABC transporter, integral membrane protein	-	2,30	3,6E-07
SMU.459	putative ABC transporter, amino acid binding protein	-	-1,93	3,6E-08
SMU.460	putative amino acid ABC transporter, permease	-	-2,00	9,3E-09
SMU.524	putative ABC transporter, ATP-binding protein	-	-1,73	4,2E-06
SMU.806c	putative glutamine ABC transporter, permease protein	-	-1,72	4,0E-06

SMU.880	multiple sugar-binding ABC transporter, permease protein MsmG	msmG	1,77	1,1E-06
SMU.882	multiple sugar-binding ABC transporter, ATP-binding protein, MsmK	msmK	2,00	2,5E-06
SMU.933	putative amino acid ABC transporter, periplasmic amino acid-binding protein	-	-2,71	2,3E-04
SMU.934	putative amino acid ABC transporter, permease protein	-	-2,02	3,3E-03
SMU.935	putative amino acid ABC transporter, permease protein	-	-1,88	4,8E-03
SMU.1035	putative ABC transporter, ATP-binding protein	glrA	-1,87	3,1E-06
SMU.1063	putative ABC transporter, ATP-binding protein, proline/glycine betaine transport system	opuAa	-1,91	2,3E-07
SMU.1067c	putative ABC transporter, permease protein	-	1,70	1,3E-04
SMU.1095	putative choline ABC transporter, osmoprotectant binding protein	opuBc	-1,73	1,1E-07
SMU.1096	putative ABC transporter, ATP-binding protein, choline transporter	opuBa	-1,82	1,3E-07
SMU.1315c	putative ATP-binding protein	-	-2,00	6,4E-08
SMU.1412c	putative ABC transporter, membrane protein subunit and ATP-binding protein	-	-2,07	1,3E-06
SMU.1667	putative branched chain amino acid ABC transporter, permease protein	livM	-1,78	1,2E-07
SMU.1668	putative branched chain amino acid ABC transporter, permease protein	livH	-1,90	7,9E-08
SMU.1669	putative ABC transporter, branched chain amino acid-binding protein	livK	-1,86	2,4E-07
SMU.1881c	putative ABC transporter, ATP-binding protein	-	-2,55	3,6E-09
SMU.1899	putative ABC transporter, ATP-binding and permease protein (fragment)	-	2,24	2,7E-02
Alanine, aspartate and glutamate metabolism (3)				
SMU.365	glutamate synthase (large subunit)	gltA	2,31	3,4E-05
SMU.366	glutamate synthase subunit beta	gltD	2,90	6,7E-06
SMU.913	glutamate dehydrogenase	-	-2,51	2,4E-07
Amino sugar and nucleotide sugar metabolism (3)				
SMU.1429	putative UDP-N-acetylmuramyl tripeptide synthetase MurC	murC2	-1,73	2,2E-06
SMU.1437	putative UDP-N-acetylglucosamine 2-epimerase	epsC	-2,45	5,6E-08
SMU.1635	putative UDP-N-acetylglucosamine pyrophosphorylase	glmU	-2,09	9,9E-08
Arginine and proline metabolism (5)				
SMU.663	N-acetyl-gamma-glutamyl-phosphate reductase	argC	1,92	1,7E-03
SMU.664	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein	argJ	1,75	2,0E-03
SMU.665	acetylglutamate kinase	argB	2,39	1,6E-04
SMU.666	acetylornithine aminotransferase	argD	2,45	3,0E-03
SMU.1218	amidase	nylA	1,79	2,4E-06
Bacteriocin related (2)				
SMU.1788c	putative bacterocin transport accessory protein, Bta	-	-3,02	3,6E-09
SMU.1889c	hypothetical protein	-	2,13	1,3E-06
Biosynthesis of secondary metabolites (5)				
SMU.937	putative mevalonate diphosphate decarboxylase	-	-1,96	5,8E-08
SMU.938	putative phosphomevalonate kinase	-	-1,70	1,0E-06
SMU.942	putative hydroxymethylglutaryl-CoA reductase	mvaA	-2,46	2,5E-06
SMU.943c	putative hydroxymethylglutaryl-CoA synthase	-	-2,93	1,7E-07
SMU.1996	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	ipk	-1,79	2,6E-07
Cell Cycle (4)				
SMU.172	putative cell growth regulatory protein	-	2,04	1,8E-06

SMU.551	cell division protein FtsA	ftsA	-1,74	1,3E-06
SMU.713	putative cell division protein FtsW	ftsW	-2,05	9,9E-08
SMU.1279c	putative cell division protein (cell shape determining protein)	-	-2,71	4,8E-09
Citrate cycle (TCA cycle) (5)				
SMU.180	putative oxidoreductase; fumarate reductase	-	2,25	2,3E-08
SMU.670	aconitate hydratase	citB	1,85	2,2E-04
SMU.671	citrate synthase	citZ	2,89	4,9E-05
SMU.672	isocitrate dehydrogenase	idh	2,83	3,1E-05
SMU.1022	2'-(5"-triphosphoribosyl)-3'-dephospho-CoA:apo-citrate lyase	citX	1,70	1,7E-05
Cofactors and Vitamins (8)				
SMU.320	putative 5-formyltetrahydrofolate cyclo-ligase	-	-1,72	6,9E-07
SMU.464	nicotinate phosphoribosyltransferase	-	-1,72	1,4E-07
SMU.841	putative aminotransferase	-	-3,06	1,6E-08
SMU.842	thiamine biosynthesis protein ThiI	thiI	-2,38	1,8E-08
SMU.947	putative dihydrofolate reductase	dfrA	-2,59	2,4E-08
SMU.954	pyridoxamine kinase	-	-3,22	2,8E-06
SMU.1074	phosphopantothenate--cysteine ligase	-	-2,06	1,1E-07
SMU.1799	nicotinic acid mononucleotide adenylyltransferase	nadD	-1,84	5,3E-08
Competence (1)				
SMU.498	putative late competence protein	comF	2,15	3,7E-02
Cysteine and methionine metabolism (2)				
SMU.496	putative cysteine synthetase A; O-acetylserine lyase	cysK	-1,74	3,0E-05
SMU.1748	aspartate kinase	akh	-1,77	6,6E-08
Energy metabolism (6)				
SMU.1098c	putative oxidoreductase	-	-2,68	1,1E-07
SMU.1473c	putative oxidoreductase	-	-2,25	2,1E-08
SMU.1531	F0F1 ATP synthase subunit delta	atpE	-1,73	5,1E-08
SMU.1532	F0F1 ATP synthase subunit B	atpF	-1,96	2,5E-08
SMU.1533	F0F1 ATP synthase subunit A	atpG	-2,06	5,6E-07
SMU.1534	F0F1 ATP synthase subunit C	atpH	-2,23	8,3E-07
Folding, Sorting and Degradation (10)				
SMU.80	heat-inducible transcription repressor	hrcA	1,87	2,3E-07
SMU.81	heat shock protein GrpE (HSP-70 cofactor)	grpE	1,96	7,5E-07
SMU.82	molecular chaperone DnaK	dnaK	2,14	6,6E-08
SMU.83	heat shock protein DnaJ (HSP-40)	dnaJ	2,04	3,0E-07
SMU.188c	Hsp33-like chaperonin	hslO	-2,06	1,9E-08
SMU.949	ATP-dependent protease ATP-binding subunit ClpX	clpX	-2,24	3,1E-08
SMU.956	putative Clp-like ATP-dependent protease, ATP-binding subunit	clp	2,12	1,8E-07
SMU.1438c	putative Zn-dependent protease	-	-3,20	7,5E-08
SMU.1787c	preprotein translocase subunit YajC	yajC	-2,37	7,4E-07
SMU.1954	chaperonin GroEL	groEL	1,98	9,2E-08
Galactose metabolism (5)				
SMU.887	galactose-1-phosphate uridylyltransferase	galT	1,80	1,2E-07
SMU.1493	tagatose 1,6-diphosphate aldolase	lacD	2,74	1,1E-02
SMU.1494	tagatose-6-phosphate kinase	lacC	3,29	5,4E-03

SMU.1495	galactose-6-phosphate isomerase subunit LacB	lacB	5,29	2,2E-04
SMU.1496	galactose-6-phosphate isomerase subunit LacA	lacA	3,73	1,1E-02
Glutathione metabolism (3)				
SMU.140	putative glutathione reductase	-	-2,12	4,9E-07
SMU.838	glutathione reductase	gshR	-1,83	2,5E-07
SMU.1296	putative glutathione S-transferase YghU	-	-3,97	2,3E-08
Glycolysis / Gluconeogenesis (6)				
SMU.494	fructose-6-phosphate aldolase	-	2,39	2,0E-07
SMU.676	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	gapN	-2,02	6,6E-08
SMU.715	triosephosphate isomerase	tpiA	-1,82	3,0E-08
SMU.871	putative fructose-1-phosphate kinase	pfkB	-1,89	3,4E-08
SMU.1421	branched-chain alpha-keto acid dehydrogenase subunit E2	pdhC	2,09	3,8E-03
SMU.1422	putative pyruvate dehydrogenase E1 component beta subunit)	pdhB	2,31	2,6E-02
Histidine metabolism (1)				
SMU.1261c	putative phosphoribosyl-ATP pyrophosphohydrolase	-	1,70	2,7E-06
Hypothetical proteins (112)				
SMU.11	hypothetical protein	-	1,71	4,1E-03
SMU.31	hypothetical protein	-	2,07	7,1E-07
SMU.33	hypothetical protein	-	2,01	2,7E-05
SMU.36	hypothetical protein	-	2,00	1,9E-05
SMU.63c	hypothetical protein	-	-1,72	3,9E-06
SMU.141	hypothetical protein	-	-2,05	2,1E-07
SMU.145	hypothetical protein	-	-2,05	1,4E-05
SMU.166	hypothetical protein	-	1,89	6,0E-07
SMU.167	hypothetical protein	-	1,83	1,5E-06
SMU.175	hypothetical protein	-	2,99	1,8E-03
SMU.176	hypothetical protein	-	2,33	1,5E-04
SMU.179	hypothetical protein	-	2,17	6,3E-08
SMU.193c	hypothetical protein	-	2,30	1,3E-02
SMU.212c	hypothetical protein	-	2,72	4,6E-02
SMU.216c	hypothetical protein	-	2,40	2,8E-02
SMU.260	hypothetical protein	-	-1,80	2,1E-06
SMU.277	hypothetical protein	-	-2,62	1,1E-07
SMU.278	hypothetical protein	-	-2,44	7,5E-09
SMU.279	hypothetical protein	-	-2,59	2,1E-07
SMU.281	hypothetical protein	-	-2,01	1,2E-06
SMU.285	hypothetical protein	-	-2,09	1,0E-05
SMU.321	hypothetical protein	-	-1,74	7,5E-08
SMU.368c	hypothetical protein	-	-1,74	3,9E-08
SMU.369c	hypothetical protein	-	-2,19	2,8E-08
SMU.381c	hypothetical protein	-	-2,39	9,8E-09
SMU.384	hypothetical protein	-	-1,86	1,0E-06
SMU.391c	hypothetical protein	-	-1,88	9,6E-05
SMU.392c	hypothetical protein	-	-1,76	2,6E-08
SMU.399	hypothetical protein	-	-3,83	1,1E-10

SMU.404c	hypothetical protein	-	1,75	3,5E-06
SMU.409	hypothetical protein	-	-2,07	6,6E-07
SMU.440	hypothetical protein	-	1,85	2,6E-07
SMU.442	hypothetical protein	-	1,70	1,4E-06
SMU.448	hypothetical protein	-	1,83	7,8E-06
SMU.475	hypothetical protein	-	6,23	2,9E-10
SMU.513	hypothetical protein	-	-1,72	1,2E-06
SMU.516	hypothetical protein	-	-1,70	2,4E-07
SMU.530c	hypothetical protein	-	-2,01	7,9E-06
SMU.571	hypothetical protein	-	1,74	3,2E-07
SMU.574c	hypothetical protein	-	2,27	7,7E-03
SMU.575c	hypothetical protein	-	2,49	9,2E-03
SMU.673	hypothetical protein	-	2,02	7,9E-05
SMU.782	hypothetical protein	-	1,71	5,8E-07
SMU.799c	hypothetical protein	-	1,84	6,6E-03
SMU.804	hypothetical protein	-	1,71	2,1E-04
SMU.844	hypothetical protein	-	1,79	1,7E-06
SMU.845	hypothetical protein	-	2,07	3,8E-07
SMU.911c	hypothetical protein	-	-2,27	1,5E-08
SMU.929c	hypothetical protein	-	-1,90	1,5E-06
SMU.932	hypothetical protein	-	-4,07	5,6E-05
SMU.948	hypothetical protein	-	-2,20	7,2E-08
SMU.955	hypothetical protein	-	-3,70	8,4E-07
SMU.984	hypothetical protein	-	2,33	8,4E-06
SMU.1048	hypothetical protein	-	-2,33	1,7E-07
SMU.1056	hypothetical protein	-	1,80	2,2E-04
SMU.1070c	hypothetical protein	-	1,85	1,7E-05
SMU.1131c	hypothetical protein	-	1,72	3,5E-05
SMU.1140c	hypothetical protein	-	-1,89	3,3E-08
SMU.1141c	hypothetical protein	-	-1,94	1,1E-07
SMU.1245c	hypothetical protein	-	1,81	1,7E-06
SMU.1249c	hypothetical protein	-	-2,01	4,0E-05
SMU.1250c	hypothetical protein	-	-1,99	5,6E-06
SMU.1262c	hypothetical protein	-	1,79	7,3E-05
SMU.1297	hypothetical protein	-	-2,08	5,8E-08
SMU.1314	hypothetical protein	-	-1,72	2,1E-07
SMU.1316c	hypothetical protein	-	-4,19	6,3E-09
SMU.1317c	hypothetical protein	-	-3,79	9,2E-08
SMU.1336	hypothetical protein	pksD	1,70	8,1E-03
SMU.1372c	hypothetical protein	-	1,74	1,4E-04
SMU.1373c	hypothetical protein	-	2,30	7,6E-05
SMU.1390	hypothetical protein	-	1,79	2,0E-05
SMU.1397c	hypothetical protein	-	-1,95	7,6E-05
SMU.1435c	hypothetical protein	-	-1,78	1,3E-05
SMU.1436c	hypothetical protein	-	-2,04	3,8E-07

SMU.1453c	hypothetical protein	-	1,70	1,4E-06
SMU.1456c	hypothetical protein	-	2,52	3,7E-07
SMU.1475c	hypothetical protein	-	-3,24	1,4E-09
SMU.1479	hypothetical protein	-	-1,74	1,1E-05
SMU.1488c	hypothetical protein	-	1,78	1,4E-02
SMU.1628	hypothetical protein	-	1,73	2,0E-06
SMU.1634c	hypothetical protein	-	-1,71	2,9E-07
SMU.1642c	hypothetical protein	-	1,96	7,5E-07
SMU.1643c	hypothetical protein	-	1,88	9,9E-06
SMU.1683c	hypothetical protein	-	1,83	9,4E-06
SMU.1703c	hypothetical protein	-	-2,08	2,8E-02
SMU.1753c	hypothetical protein	-	2,01	2,3E-06
SMU.1754c	hypothetical protein	-	1,96	5,7E-06
SMU.1755c	hypothetical protein	-	2,08	9,5E-06
SMU.1757c	hypothetical protein	-	1,95	1,3E-05
SMU.1758c	hypothetical protein	-	2,12	8,8E-06
SMU.1760c	hypothetical protein	-	2,04	7,2E-06
SMU.1761c	hypothetical protein	-	2,26	1,3E-06
SMU.1762c	hypothetical protein	-	2,09	5,7E-06
SMU.1763c	hypothetical protein	-	1,88	2,0E-06
SMU.1764c	hypothetical protein	-	1,95	7,8E-08
SMU.1782	hypothetical protein	-	-2,28	2,2E-07
SMU.1800c	hypothetical protein	-	-1,90	5,3E-08
SMU.1802c	hypothetical protein	-	-2,38	2,1E-07
SMU.1803c	hypothetical protein	-	1,73	1,8E-04
SMU.1861c	hypothetical protein	-	-1,90	7,6E-06
SMU.1882c	hypothetical protein	-	-3,63	2,0E-07
SMU.1900	hypothetical protein	-	1,78	5,0E-05
SMU.1940c	hypothetical protein	-	-1,89	2,9E-07
SMU.1951c	hypothetical protein	-	2,15	3,1E-06
SMU.1956c	hypothetical protein	-	2,37	1,9E-07
SMU.1975c	hypothetical protein	-	2,11	3,2E-07
SMU.1976c	hypothetical protein	-	1,98	2,8E-07
SMU.1999c	hypothetical protein	-	-2,65	1,6E-07
SMU.2046c	hypothetical protein	-	1,90	7,5E-07
SMU.2077c	hypothetical protein	-	-1,83	2,1E-07
SMU.2096c	hypothetical protein	-	1,81	3,5E-05
SMU.2129c	hypothetical protein	-	-2,02	2,4E-06
Lipid metabolism (3)				
SMU.495	glycerol dehydrogenase	gldA	2,54	1,3E-07
SMU.1744	3-oxoacyl-(acyl carrier protein) synthase III	fabH	-2,34	5,5E-08
SMU.1746c	enoyl-CoA hydratase	-	-1,77	7,0E-07
miscellaneous (20)				
SMU.173	putative ppGpp-regulated growth inhibitor	-	2,14	1,2E-06
SMU.400	putative secreted esterase	-	-3,37	1,4E-08

SMU.609	putative 40K cell wall protein precursor	-	2,68	5,9E-07
SMU.610	cell surface antigen SpaP	spaP	-2,62	1,3E-09
SMU.707c	putative endolysin	-	-2,09	5,0E-09
SMU.924	thiol peroxidase	tpx	-2,49	1,2E-08
SMU.981	putative BglB fragment	bglB1	1,74	4,5E-02
SMU.1213c	putative 5'-nucleotidase precursor	-	1,71	6,6E-06
SMU.1243	putative low temperature requirement A protein	-	-3,08	6,9E-06
SMU.1294	flavodoxin	flaW	-2,57	6,7E-08
SMU.1334	putative phosphopantetheinyl transferase	sfp	1,71	1,0E-02
SMU.1396	glucan-binding protein C, GbpC	gbpC	-1,84	9,1E-04
SMU.1474c	ribonuclease Z	-	-2,76	1,4E-08
SMU.1476c	putative GTP-binding protein	-	-3,38	3,0E-07
SMU.1645	tellurite resistance protein TehB	-	-1,96	6,0E-08
SMU.1693	putative hemolysin	hlyX	-1,85	2,3E-07
SMU.1702c	putative phosphatase	-	-1,97	3,5E-02
SMU.1801c	GTP-binding protein YqeH	-	-2,16	1,7E-07
SMU.1816c	putative maturase-related protein	-	1,82	2,5E-02
SMU.1930	putative cytoplasmic membrane protein; LemA-like protein	lemA	-2,60	8,2E-07
Mobile and extrachromosomal elements (7)				
SMU.149	putative transposase	-	1,86	2,7E-04
SMU.1032	putative transposon integrase; Tn916 ORF3-like	tnr5	-1,85	1,9E-06
SMU.1351	putative putative transposase	-	1,89	5,2E-08
SMU.1352	putative transposase	-	1,97	3,1E-06
SMU.1353	putative transposase	-	1,70	7,4E-07
SMU.1818c	transposase	-	2,19	4,6E-03
SMU.1888	putative transposase	-	1,93	1,6E-03
Nitrogen metabolism (1)				
SMU.1595	putative carbonic anhydrase precursor	cah	-1,76	6,6E-06
Phenylalanine, tyrosine and tryptophan biosynthesis (2)				
SMU.1836	phospho-2-dehydro-3-deoxyheptonate aldolase	aroG	-2,31	2,0E-03
SMU.1837	phospho-2-dehydro-3-deoxyheptonate aldolase	aroH	-24,86	5,5E-04
Phosphotransferase system (PTS) (8)				
SMU.1185	PTS system, mannitol-specific enzyme IIBC component	mtlA1	2,89	1,8E-06
SMU.1491	PTS system, lactose-specific enzyme IIBC EIIBC-LAC)	lacE	2,13	4,1E-03
SMU.1492	PTS system, lactose-specific enzyme IIA EIIA-LAC)	lacF	3,34	7,5E-04
SMU.1879	putative PTS system, mannose-specific component IID	-	1,79	5,6E-07
SMU.1957	putative PTS system, mannose-specific IID component	-	2,41	1,3E-06
SMU.1958c	putative PTS system, mannose-specific IIC component	-	2,59	1,2E-07
SMU.1960c	putative PTS system, mannose-specific IIB component	-	2,42	1,7E-08
SMU.1961c	putative PTS system, sugar-specific enzyme IIA component	-	1,95	1,1E-07
Purine metabolism (8)				
SMU.32	amidophosphoribosyltransferase	purF	1,96	4,7E-06
SMU.34	phosphoribosylaminoimidazole synthetase	purM	2,12	5,4E-06
SMU.35	phosphoribosylglycinamide formyltransferase	purN	2,01	1,6E-05
SMU.37	bifunctional phosphoribosylaminoimidazolecarboxamide	purH	2,15	2,2E-05

	formyltransferase/IMP cyclohydrolase			
SMU.155	polynucleotide phosphorylase/polyadenylase	pnpA	-2,50	2,9E-08
SMU.668c	ribonucleotide-diphosphate reductase subunit alpha	-	-1,89	5,5E-06
SMU.1295	adenosine deaminase	add	-2,88	2,7E-08
SMU.1467	adenine phosphoribosyltransferase	apt	-2,52	1,5E-07
Pyrimidine metabolism (8)				
SMU.463	putative thioredoxin reductase (NADPH)	trxB	-2,24	2,2E-08
SMU.595	dihydroorotate dehydrogenase 1A	pyrD	-2,12	6,4E-08
SMU.859	carbamoyl phosphate synthase small subunit	pyrA	1,74	2,7E-06
SMU.860	carbamoyl phosphate synthase large subunit	carB	1,83	3,6E-06
SMU.869	putative thioredoxin reductase	trxB2	-2,06	1,4E-08
SMU.944	thymidylate synthase	thyA	-3,15	1,8E-08
SMU.1086	thymidine kinase	kitH	-2,39	1,2E-07
SMU.1869	putative thioredoxin	trxA	-1,87	2,3E-05
Pyruvate metabolism (5)				
SMU.137	malate dehydrogenase	mleS	-5,58	1,5E-09
SMU.139	oxalate decarboxylase	-	-2,21	1,7E-06
SMU.490	putative pyruvate formate-lyase activating enzyme	pflC	2,19	5,5E-04
SMU.493	formate acetyltransferase (pyruvate formate-lyase 2)	pfl2	2,30	3,6E-07
SMU.1299c	putative acetate kinase	-	1,81	5,4E-07
Replication and repair (12)				
SMU.60	DNA alkylation repair enzyme	-	1,71	3,2E-06
SMU.895	DNA-damage-inducible protein	-	1,99	2,2E-06
SMU.897	putative type I restriction-modification system, helicase subunits	-	2,11	7,5E-07
SMU.1002	DNA topoisomerase I	topA	-2,24	1,9E-07
SMU.1313c	bifunctional ATP-dependent DNA helicase/DNA polymerase III subunit epsilon	-	-2,00	7,4E-07
SMU.1472	putative single-strand DNA-specific exonuclease RecJ	recJ	-2,05	2,0E-08
SMU.1851	excinuclease ABC subunit A	uvrA	-1,99	4,6E-07
SMU.1873	ribonuclease HIII	rnh3	-2,02	3,7E-08
SMU.1990	DNA-directed RNA polymerase subunit beta	rpoB	-1,95	3,5E-08
SMU.2001	DNA-directed RNA polymerase subunit alpha	rpoA	-1,92	7,0E-08
SMU.2078c	Holliday junction resolvase-like protein	-	-1,72	2,2E-07
SMU.2089	DNA mismatch repair protein	mutL	-1,76	1,1E-06
Signal transduction (3)				
SMU.1916	putative histidine kinase of the competence regulon, ComD	comD	-2,48	5,5E-09
SMU.1917	putative response regulator of the competence regulon, ComE; response regulator of sakacin A production	comE	-2,94	8,0E-10
SMU.1965c	putative histidine kinase	-	-2,06	2,8E-06
Starch and sucrose metabolism (5)				
SMU.79	fructan hydrolase; exo-beta-D-fructosidase; FruB	fruB	2,32	1,3E-05
SMU.881	sucrose phosphorylase, GtfA	gtfA	1,78	7,4E-06
SMU.883	dextran glucosidase DexB	dexB	1,96	4,9E-06
SMU.1004	glucosyltransferase-I	gtfB	-2,24	1,5E-06
SMU.1005	glucosyltransferase-Si	gtfC	-2,92	2,0E-08

Transcription (22)				
SMU.136c	putative transcriptional regulator	-	2,12	3,8E-07
SMU.168	putative transcriptional regulator	-	1,88	2,1E-07
SMU.410	putative transcriptional regulator	brpA	-1,79	1,5E-06
SMU.441	putative transcriptional regulator	-	1,80	5,3E-07
SMU.491	putative DeoR-type transcriptional regulator	-	1,83	2,4E-05
SMU.870	putative transcriptional regulator of sugar metabolism	-	-2,14	2,0E-08
SMU.921	putative transcriptional regulator	-	-1,90	2,2E-06
SMU.930c	putative transcriptional regulator	-	-2,48	1,6E-06
SMU.977	putative transcriptional antiterminator LicT (fragment)	licT	1,75	1,6E-05
SMU.1097c	putative transcriptional regulator protein	-	-2,02	9,4E-08
SMU.1142c	transcriptional regulator Spx	spxA	-2,06	3,7E-07
SMU.1246c	putative transcriptional regulator	-	1,88	1,6E-05
SMU.1409c	putative transcriptional regulator	-	1,90	9,8E-08
SMU.1498	lactose repressor	lacR	2,76	2,1E-08
SMU.1566	putative maltose operon transcriptional repressor	malR	-2,36	4,2E-07
SMU.1657c	putative nitrogen regulatory protein PII	-	3,30	3,8E-06
SMU.1745c	putative transcriptional regulator	-	-2,49	6,4E-07
SMU.1824c	transcriptional repressor CodY	-	-1,75	1,8E-07
SMU.1923c	transcriptional regulator NrdR	nrdR	-1,77	8,9E-07
SMU.1947	transcription antitermination protein NusG	nusG	-2,62	8,8E-09
SMU.2044	putative stringent response protein, ppGpp synthetase	relA	-1,88	3,9E-07
SMU.2084c	transcriptional regulator Spx	spxA	1,70	4,1E-07
Translation (18)				
SMU.91	trigger factor	tig	-2,31	7,0E-07
SMU.120	50S ribosomal protein L28	rpmB	-2,01	3,2E-05
SMU.500	putative ribosome-associated protein	-	2,15	2,6E-07
SMU.950	ribosome biogenesis GTP-binding protein YsxC	engB	-1,88	9,8E-08
SMU.1003	tRNA (uracil-5-)-methyltransferase Gid	gid	-1,84	4,5E-07
SMU.1085	peptide chain release factor 1	prfA	-1,95	4,4E-07
SMU.1200	30S ribosomal protein S1	rpsA	-1,98	1,6E-08
SMU.1298	50S ribosomal protein L31 type B	rpmE2	-1,79	1,8E-05
SMU.1477	tRNA delta(2)-isopentenylpyrophosphate transferase	miaA	-2,06	1,3E-07
SMU.1610	50S ribosomal protein L33	rpmG	1,74	4,2E-07
SMU.1943	leucyl-tRNA synthetase	leuS	-2,62	6,4E-09
SMU.1950	putative pseudouridylate synthase	-	-1,90	2,5E-07
SMU.1992	tyrosyl-tRNA synthetase	tyrS	-2,07	5,7E-07
SMU.2000	50S ribosomal protein L17	rplQ	-1,80	1,4E-06
SMU.2002	30S ribosomal protein S11	rs11	-1,83	5,4E-08
SMU.2003	30S ribosomal protein S13	rpsM	-1,97	7,2E-08
SMU.2003a	50S ribosomal protein L36	rpmJ	-1,70	4,2E-06
SMU.2004	translation initiation factor IF-1	infA	-1,76	3,2E-06
Transporter (12)				
SMU.89c	putative nitrite transporter	-	-2,41	3,3E-08
SMU.138	putative malate permease	-	-4,21	3,9E-08

SMU.396	putative glycerol uptake facilitator protein	glpF	-2,52	1,1E-08
SMU.946	putative permease	-	-2,78	2,1E-07
SMU.951	putative amino acid permease	-	-2,19	2,1E-08
SMU.1100c	putative permease	-	-1,72	6,3E-08
SMU.1176	putative cation efflux transporter	-	-2,89	5,5E-09
SMU.1658	putative ammonium transporter, NrgA protein	nrgA	2,73	2,9E-05
SMU.1708	potassium transporter peripheral membrane component	trkA	-1,94	3,1E-07
SMU.1942c	putative amino acid binding protein	-	-2,90	1,2E-06
SMU.1966c	putative periplasmic sugar-binding protein	-	-2,35	2,4E-07
SMU.2059c	putative integral membrane protein	-	-4,33	5,1E-08
Valine, leucine and isoleucine biosynthesis (1)				
SMU.1381	isopropylmalate isomerase small subunit	leuD	1,81	1,5E-05

8.3 **Supplementary table 3**

Supplementary table S3.

Genes that were significant changed compared to the wildtype. Significant changes (fold change > ±1.7; P-value < 0.05) are marked in bold.

Δ*luxS*$_2$SahH **up-regulated genes: 87 (control) , 88 (10 nM AI-2), 76 (25 µM AI-2)** **down-regulated genes: 49 (control), 39 (10 nM AI-2), 40 (25 µM AI-2)**					
Locus tag	**Condition**	**description**	**Gene symbol**	**Fold change**	**P-value**
ABC transporters (9, 8, 8)					
SMU.426	sahH	copper-transporting ATPase; P-type ATPase	copA	-2,26	9,6E-08
SMU.426	SahH 10 nM	copper-transporting ATPase; P-type ATPase	copA	-2,30	8,5E-08
SMU.426	SahH 25 µM	copper-transporting ATPase; P-type ATPase	copA	-2,29	8,7E-08
SMU.524	sahH	putative ABC transporter, ATP-binding protein	-	-1,82	2,4E-06
SMU.524	SahH 10 nM	putative ABC transporter, ATP-binding protein	-	-1,83	2,2E-06
SMU.524	SahH 25 µM	putative ABC transporter, ATP-binding protein	-	-1,81	2,5E-06
SMU.731	sahH	putative ABC transporter, ATP-binding protein	-	-1,83	1,2E-06
SMU.731	SahH 10 nM	putative ABC transporter, ATP-binding protein	-	-1,73	2,1E-06
SMU.731	SahH 25 µM	putative ABC transporter, ATP-binding protein	-	-1,71	2,5E-06
SMU.803c	sahH	putative ABC transporter, ATP-binding protein	-	2,13	7,5E-03
SMU.803c	SahH 10 nM	putative ABC transporter, ATP-binding protein	-	2,14	7,2E-03
SMU.934	SahH 25 µM	putative amino acid ABC transporter, permease protein	-	1,78	8,9E-03
SMU.935	SahH 25 µM	putative amino acid ABC transporter, permease protein	-	1,73	9,2E-03
SMU.936	SahH 10 nM	putative amino acid ABC transporter, ATP-binding protein	-	1,76	7,8E-03
SMU.936	SahH 25 µM	putative amino acid ABC transporter, ATP-binding protein	-	1,87	4,7E-03
SMU.1067c	sahH	putative ABC transporter, permease protein	-	2,26	1,0E-05
SMU.1067c	SahH 10 nM	putative ABC transporter, permease protein	-	2,55	4,5E-06

SMU.1067c	SahH 25 µM	putative ABC transporter, permease protein	-	2,58	4,1E-06
SMU.1068c	sahH	putative ABC transporter, ATP-binding protein	-	2,16	1,8E-05
SMU.1068c	SahH 10 nM	putative ABC transporter, ATP-binding protein	-	2,42	7,9E-06
SMU.1068c	SahH 25 µM	putative ABC transporter, ATP-binding protein	-	2,57	5,2E-06
SMU.1094	sahH	putative ABC transporter, ATP-binding protein	-	-1,71	4,3E-04
SMU.1563	sahH	putative cation-transporting P-type ATPase PacL	pacL	-1,73	3,7E-07
SMU.1899	sahH	putative ABC transporter, ATP-binding and permease protein (fragment)	-	2,43	1,9E-02
SMU.1899	SahH 10 nM	putative ABC transporter, ATP-binding and permease protein (fragment)	-	2,26	2,6E-02
Bacteriocin related (6, 5, 6)					
SMU.151	sahH	hypothetical protein	-	1,91	7,9E-04
SMU.151	SahH 10 nM	hypothetical protein	-	2,09	3,9E-04
SMU.151	SahH 25 µM	hypothetical protein	-	1,87	9,3E-04
SMU.1895c	sahH	hypothetical protein	-	2,20	1,9E-06
SMU.1895c	SahH 25 µM	hypothetical protein	-	2,14	2,4E-06
SMU.1896c	sahH	hypothetical protein	-	2,07	1,1E-06
SMU.1896c	SahH 10 nM	hypothetical protein	-	1,88	2,7E-06
SMU.1896c	SahH 25 µM	hypothetical protein	-	2,04	1,3E-06
SMU.1902c	sahH	hypothetical protein	-	2,14	1,7E-05
SMU.1902c	SahH 10 nM	hypothetical protein	-	2,78	2,8E-06
SMU.1902c	SahH 25 µM	hypothetical protein	-	2,44	6,5E-06
SMU.1905c	sahH	putative bacteriocin secretion protein	-	1,81	1,4E-04
SMU.1905c	SahH 10 nM	putative bacteriocin secretion protein	-	1,84	1,2E-04
SMU.1905c	SahH 25 µM	putative bacteriocin secretion protein	-	1,89	9,3E-05
SMU.1913c	sahH	putative immunity protein, BLpL-like	-	1,74	1,1E-04
SMU.1913c	SahH 10 nM	putative immunity protein, BLpL-like	-	1,87	5,4E-05
SMU.1913c	SahH 25 µM	putative immunity protein, BLpL-like	-	1,85	6,0E-05
Biosynthesis of secondary metabolites (1, 0, 1)					
SMU.1343c	SahH 25 µM	putative polyketide synthase	-	1,77	1,7E-03
SMU.1457	sahH	putative dTDP-glucose-4,6-dehydratase	rmlB	1,70	5,8E-08
Citrate cycle (TCA cycle) (3, 3, 3)					
SMU.670	sahH	aconitate hydratase	citB	-3,23	4,8E-06
SMU.670	SahH 10 nM	aconitate hydratase	citB	-2,94	8,2E-06
SMU.670	SahH 25 µM	aconitate hydratase	citB	-2,77	1,1E-05
SMU.671	sahH	citrate synthase	citZ	-3,22	2,7E-05
SMU.671	SahH 10 nM	citrate synthase	citZ	-3,29	2,4E-05
SMU.671	SahH 25 µM	citrate synthase	citZ	-3,16	3,0E-05
SMU.672	sahH	isocitrate dehydrogenase	idh	-2,97	2,4E-05
SMU.672	SahH 10 nM	isocitrate dehydrogenase	idh	-3,07	2,0E-05
SMU.672	SahH 25 µM	isocitrate dehydrogenase	idh	-2,93	2,6E-05
Competence (4, 3, 0)					
SMU.498	SahH 10 nM	putative late competence protein	comF	2,56	1,7E-02
SMU.499	sahH	putative late competence protein	-	1,75	4,0E-03
SMU.499	SahH 10 nM	putative late competence protein	-	1,79	3,3E-03

SMU.625	sahH	putative competence protein	comEA	1,81	3,4E-03
SMU.625	SahH 10 nM	putative competence protein	comEA	2,01	1,5E-03
SMU.1001	sahH	putative DNA processing Smf protein	smf	2,46	3,3E-02
SMU.1259	sahH	restriction endonuclease	-	1,94	3,6E-02
Cysteine and methionine metabolism (1, 1, 1)					
SMU.474	sahH	S-ribosylhomocysteinase	luxS	-140,64	9,6E-05
SMU.474	SahH 10 nM	S-ribosylhomocysteinase	luxS	-149,68	8,9E-05
SMU.474	SahH 25 µM	S-ribosylhomocysteinase	luxS	-64,75	2,6E-04
Energy metabolism (2, 2, 2)					
SMU.1040c	sahH	putative oxidoreductase, short-chain dehydrogenase/reductase	-	-1,77	5,2E-08
SMU.1040c	SahH 10 nM	putative oxidoreductase, short-chain dehydrogenase/reductase	-	-1,80	4,3E-08
SMU.1040c	SahH 25 µM	putative oxidoreductase, short-chain dehydrogenase/reductase	-	-1,84	3,4E-08
SMU.1098c	sahH	putative oxidoreductase	-	-1,91	1,4E-06
SMU.1098c	SahH 10 nM	putative oxidoreductase	-	-1,85	1,9E-06
SMU.1098c	SahH 25 µM	putative oxidoreductase	-	-1,84	2,0E-06
Fatty acid biosynthesis (0, 0, 2)					
SMU.1335c	SahH 25 µM	putative enoyl-(acyl-carrier-protein) reductase	-	1,71	1,3E-02
SMU.1344c	SahH 25 µM	putative malonyl-CoA acyl-carrier-protein transacylase	-	1,77	9,9E-04
Folding, Sorting and Degradation (2, 2, 2)					
SMU.956	sahH	putative Clp-like ATP-dependent protease, ATP-binding subunit	clp	-1,94	4,1E-07
SMU.956	SahH 10 nM	putative Clp-like ATP-dependent protease, ATP-binding subunit	clp	-2,08	2,1E-07
SMU.956	SahH 25 µM	putative Clp-like ATP-dependent protease, ATP-binding subunit	clp	-2,15	1,7E-07
SMU.2006	sahH	preprotein translocase subunit SecY	secY	2,14	1,1E-08
SMU.2006	SahH 10 nM	preprotein translocase subunit SecY	secY	1,96	2,4E-08
SMU.2006	SahH 25 µM	preprotein translocase subunit SecY	secY	2,00	2,0E-08
Galactose metabolism (2, 2, 1)					
SMU.116	sahH	tagatose 1,6-diphosphate aldolase	lacD2	2,26	4,1E-02
SMU.116	SahH 10 nM	tagatose 1,6-diphosphate aldolase	lacD2	2,36	3,4E-02
SMU.1495	sahH	galactose-6-phosphate isomerase subunit LacB	lacB	2,20	1,1E-02
SMU.1495	SahH 10 nM	galactose-6-phosphate isomerase subunit LacB	lacB	2,21	1,0E-02
SMU.1495	SahH 25 µM	galactose-6-phosphate isomerase subunit LacB	lacB	2,04	1,6E-02
Glutathione metabolism (2, 1, 1)					
SMU.140	sahH	putative glutathione reductase	-	-1,78	2,5E-06
SMU.140	sahH 10 nM	putative glutathione reductase	-	-1,83	1,9E-06
SMU.1296	sahH	putative glutathione S-transferase YghU	-	-1,84	3,5E-06
SMU.1296	SahH 25 µM	putative glutathione S-transferase YghU	-	-1,73	6,7E-06
Glyoxylate and dicarboxylate metabolism (1, 1, 1)					

SMU.139	sahH	oxalate decarboxylase	-	-1,81	9,9E-06
SMU.139	SahH 10 nM	oxalate decarboxylase		-1,87	7,4E-06
SMU.139	SahH 25 µM	oxalate decarbocylase	-	-1,74	1,5E-05
Hypothetical proteins (43, 40, 36)					
SMU.40	sahH	hypothetical protein	-	-1,79	4,1E-03
SMU.41	sahH	hypothetical protein	-	-1,78	1,5E-02
SMU.141	sahH	hypothetical protein	-	-1,71	1,3E-06
SMU.141	SahH 10 nM	hypothetical protein	-	-1,79	9,1E-06
SMU.141	SahH 25 µM	hypothetical protein	-	-1,73	1,1E-06
SMU.152	sahH	hypothetical protein	-	1,77	4,9E-06
SMU.152	SahH 10 nM	hypothetical protein	-	1,78	4,7E-06
SMU.152	SahH 25 µM	hypothetical protein	-	1,84	3,4E-06
SMU.153	sahH	hypothetical protein	-	3,71	1,5E-02
SMU.153	SahH 25 µM	hypothetical protein	-	2,63	4,7E-02
SMU.200c	sahH	hypothetical protein	-	-1,77	3,7E-06
SMU.200c	SahH 10 nM	hypothetical protein	-	-1,86	1,7E-06
SMU.205c	sahH	hypothetical protein	-	-2,20	7,9E-08
SMU.205c	SahH 10 nM	hypothetical protein	-	-2,18	8,6E-08
SMU.205c	SahH 25 µM	hypothetical protein	-	-1,92	2,6E-07
SMU.260	sahH	hypothetical protein	-	-1,71	3,7E-06
SMU.276c	SahH 25 µM	hypothetical protein	-	1,85	5,0E-03
SMU.399	sahH	hypothetical protein	-	-3,40	1,9E-10
SMU.399	SahH 10 nM	hypothetical protein	-	-3,26	2,3E-10
SMU.399	SahH 25 µM	hypothetical protein	-	-3,25	2,4E-10
SMU.475	sahH	hypothetical protein	-	11,46	4,8E-11
SMU.475	SahH 10 nM	hypothetical protein	-	11,70	4,6E-11
SMU.475	SahH 25 µM	hypothetical protein	-	11,06	5,3E-11
SMU.575c	sahH	hypothetical protein	-	2,22	1,7E-02
SMU.575c	SahH 10 nM	hypothetical protein	-	2,09	2,3E-02
SMU.658	SahH 10 nM	hypothetical protein	-	1,86	2,3E-02
SMU.658	SahH 25 µM	hypothetical protein	-	1,88	2,2E-02
SMU.673	sahH	hypothetical protein	-	-2,00	8,6E-05
SMU.673	SahH 10 nM	hypothetical protein	-	-2,02	8,0E-05
SMU.673	SahH 25 µM	hypothetical protein	-	-1,91	1,3E-04
SMU.730	sahH	hypothetical protein	-	-1,76	1,9E-06
SMU.732	sahH	hypothetical protein	-	-2,09	3,9E-07
SMU.732	SahH 10 nM	hypothetical protein	-	-1,87	7,6E-07
SMU.732	SahH 25 µM	hypothetical protein	-	-1,99	6,0E-07
SMU.734	sahH	hypothetical protein	-	-1,93	1,2E-06
SMU.734	SahH 10 nM	hypothetical protein	-	-1,86	1,1E-06
SMU.734	SahH 25 µM	hypothetical protein	-	-1,84	1,9E-06
SMU.735	sahH	hypothetical protein	-	-1,78	2,7E-06
SMU.735	SahH 25 µM	hypothetical protein	-	-1,71	4,1E-06
SMU.738	sahH	hypothetical protein	-	-1,96	4,8E-07
SMU.738	SahH 10 nM	hypothetical protein	-	-1,87	7,4E-06

SMU.738	SahH 25 µM	hypothetical protein	-	-1,84	8,9E-07
SMU.782	sahH	hypothetical protein	-	2,04	1,0E-07
SMU.782	SahH 10 nM	hypothetical protein	-	2,02	1,1E-07
SMU.782	SahH 25 µM	hypothetical protein	-	1,98	1,3E-07
SMU.798c	sahH	hypothetical protein	-	2,27	1,3E-02
SMU.798c	SahH 10 nM	hypothetical protein	-	2,07	2,1E-02
SMU.836	sahH	hypothetical protein	-	1,84	2,4E-02
SMU.836	SahH 10 nM	hypothetical protein	-	1,81	2,7E-02
SMU.941c	sahH	hypothetical protein	-	1,96	2,7E-02
SMU.941c	SahH 10 nM	hypothetical protein	-	2,59	6,0E-03
SMU.941c	SahH 25 µM	hypothetical protein	-	2,97	3,1E-03
SMU.986c	sahH	hypothetical protein	-	-1,77	1,0E-05
SMU.986c	SahH 10 nM	hypothetical protein	-	-1,80	3,1E-06
SMU.986c	SahH 25 µM	hypothetical protein	-	-1,80	8,6E-06
SMU.1069c	sahH	hypothetical protein	-	1,96	2,2E-05
SMU.1069c	SahH 10 nM	hypothetical protein	-	2,25	7,3E-06
SMU.1069c	SahH 25 µM	hypothetical protein	-	2,30	6,0E-06
SMU.1070c	sahH	hypothetical protein	-	2,05	6,3E-06
SMU.1070c	SahH 10 nM	hypothetical protein	-	2,55	1,3E-06
SMU.1070c	SahH 25 µM	hypothetical protein	-	2,57	1,2E-06
SMU.1089	sahH	hypothetical protein	-	1,74	2,3E-06
SMU.1090	sahH	hypothetical protein	-	1,85	5,6E-08
SMU.1090	SahH 10 nM	hypothetical protein	-	1,83	6,4E-08
SMU.1090	SahH 25 µM	hypothetical protein	-	1,77	8,9E-08
SMU.1205c	sahH	hypothetical protein	-	1,70	9,0E-06
SMU.1336	SahH 25 µM	hypothetical protein	pksD	1,78	5,5E-03
SMU.1378	SahH 10 nM	hypothetical protein	-	1,75	2,5E-02
SMU.1390	sahH	hypothetical protein	-	1,90	1,1E-05
SMU.1390	SahH 10 nM	hypothetical protein	-	1,82	1,6E-05
SMU.1390	SahH 25 µM	hypothetical protein	-	2,15	3,6E-06
SMU.1397c	sahH	hypothetical protein	-	-1,93	8,2E-05
SMU.1435c	sahH	hypothetical protein	-	1,73	1,9E-05
SMU.1435c	SahH 10 nM	hypothetical protein	-	1,72	2,3E-06
SMU.1488c	SahH 10 nM	hypothetical protein	-	-1,78	8,1E-07
SMU.1553c	sahH	hypothetical protein	-	1,92	4,3E-03
SMU.1553c	SahH 10 nM	hypothetical protein	-	2,17	1,8E-03
SMU.1553c	SahH 25 µM	hypothetical protein	-	2,14	2,0E-03
SMU.1574c	sahH	hypothetical protein	-	-1,81	1,6E-05
SMU.1574c	SahH 10 nM	hypothetical protein	-	-2,09	4,5E-06
SMU.1574c	SahH 25 µM	hypothetical protein	-	-1,93	9,0E-06
SMU.1703c	SahH 10 nM	hypothetical protein	-	-2,15	2,4E-02
SMU.1703c	SahH 25 µM	hypothetical protein	-	-2,16	2,3E-02
SMU.1818c	sahH	hypothetical protein	-	1,74	2,1E-02
SMU.1895c	SahH 10 nM	hypothetical protein	-	2,06	3,3E-06
SMU.1903c	SahH 10 nM	hypothetical protein	-	1,76	2,4E-04

SMU.1903c	SahH 25 µM	hypothetical protein	-	1,72	3,0E-04
SMU.1904c	sahH	hypothetical protein	-	1,78	2,8E-04
SMU.1904c	SahH 10 nM	hypothetical protein	-	1,89	1,6E-04
SMU.1904c	SahH 25 µM	hypothetical protein	-	1,92	1,4E-04
SMU.1908c	sahH	hypothetical protein	-	2,02	1,6E-04
SMU.1908c	SahH 10 nM	hypothetical protein	-	2,26	6,8E-05
SMU.1908c	SahH 25 µM	hypothetical protein	-	2,27	6,6E-05
SMU.1909c	sahH	hypothetical protein	-	1,94	2,6E-04
SMU.1909c	SahH 10 nM	hypothetical protein	-	2,16	1,1E-04
SMU.1909c	SahH 25 µM	hypothetical protein	-	2,07	1,5E-04
SMU.1910c	sahH	hypothetical protein	-	1,79	5,1E-04
SMU.1910c	SahH 10 nM	hypothetical protein	-	1,95	2,4E-04
SMU.1910c	SahH 25 µM	hypothetical protein	-	1,90	2,9E-04
SMU.1912c	sahH	hypothetical protein	-	1,74	7,2E-04
SMU.1912c	SahH 10 nM	hypothetical protein	-	1,93	2,9E-04
SMU.1912c	SahH 25 µM	hypothetical protein	-	1,95	2,7E-04
SMU.1956c	sahH	hypothetical protein	-	2,28	2,5E-07
SMU.1956c	SahH 10 nM	hypothetical protein	-	1,94	9,9E-07
SMU.1956c	SahH 25 µM	hypothetical protein	-	1,94	9,6E-07
SMU.1975c	sahH	hypothetical protein	-	1,77	1,6E-06
SMU.1975c	SahH 10 nM	hypothetical protein	-	1,71	2,0E-02
SMU.2080	sahH	hypothetical protein	-	2,08	1,8E-02
SMU.2080	SahH 10 nM	hypothetical protein	-	2,17	1,5E-02
SMU.2133c	SahH 25 µM	hypothetical protein	-	-1,90	2,5E-03
SMU.2136c	SahH 10 nM	hypothetical protein	-	1,72	7,1E-06
SMU.2136c	SahH 25 µM	hypothetical protein	-	1,72	7,5E-06
SMU.2146c	sahH	hypothetical protein	-	2,44	3,1E-06
SMU.2146c	SahH 10 nM	hypothetical protein	-	2,30	4,6E-06
SMU.2146c	SahH 25 µM	hypothetical protein	-	2,15	8,0E-06
SMU.2147c	sahH	hypothetical protein	-	2,13	2,8E-06
SMU.2147c	SahH 10 nM	hypothetical protein	-	2,21	2,1E-06
SMU.2147c	SahH 25 µM	hypothetical protein	-	2,30	1,6E-06
Miscellaneous (10, 13, 13)					
SMU.22	sahH	putative secreted antigen GbpB/SagA; putative peptidoglycan hydrolase	gbpB	1,76	5,0E-06
SMU.22	SahH 10 nM	putative secreted antigen GbpB/SagA; putative peptidoglycan hydrolase	gbpB	1,81	3,6E-06
SMU.22	SahH 25 µM	putative secreted antigen GbpB/SagA; putative peptidoglycan hydrolase	gbpB	1,73	5,8E-06
SMU.121	SahH 10 nM	putative DinF, damage-inducible protein; cation efflux pump (multidrug resistance protein)	dinF	1,99	2,4E-02
SMU.153	SahH 10 nM	hypothetical protein	-	4,20	9,9E-03
SMU.400	sahH	putative secreted esterase	-	-2,97	2,8E-08
SMU.400	SahH 10 nM	putative secreted esterase	-	-2,77	4,1E-08
SMU.400	SahH 25 µM	putative secreted esterase	-	-2,81	3,8E-08

SMU.427	sahH	putative copper chaperone	copZ	-2,08	3,1E-07
SMU.427	SahH 10 nM	putative copper chaperone	copZ	-2,06	3,4E-07
SMU.427	SahH 25 µM	putative copper chaperone	copZ	-2,09	3,0E-07
SMU.633	sahH	putative thioesterase	-	-1,82	3,4E-05
SMU.633	SahH 10 nM	putative thioesterase	-	-1,71	6,4E-05
SMU.633	SahH 25 µM	putative thioesterase	-	-1,74	5,6E-05
SMU.655	SahH 25 µM	putative MutE	mutE1	1,72	4,7E-03
SMU.764	SahH 10 nM	alkyl hydroperoxide reductase	ahpC	-1,70	2,3E-06
SMU.764	SahH 25 µM	alkyl hydroperoxide reductase	ahpC	-1,70	2,3E-06
SMU.940c	sahH	putative hemolysin III	-	1,75	2,2E-02
SMU.940c	SahH 10 nM	putative hemolysin III	-	2,28	4,0E-03
SMU.940c	SahH 25 µM	putative hemolysin III	-	2,49	2,4E-03
SMU.1091	SahH 25 µM	cell wall protein, WapE	wapE	1,82	1,9E-06
SMU.1334	SahH 25 µM	putative phosphopantetheinyl transferase	sfp	1,74	9,0E-03
SMU.1337c	sahH	alpha/beta superfamily hydrolase	-	1,88	3,2E-03
SMU.1337c	SahH 25 µM	alpha/beta superfamily hydrolase	-	2,04	1,7E-03
SMU.1345c	sahH	putative peptide synthetase MycA	-	2,34	6,1E-07
SMU.1345c	SahH 10 nM	putative peptide synthetase MycA	-	2,29	7,2E-07
SMU.1345c	SahH 25 µM	putative peptide synthetase MycA	-	2,59	3,1E-07
SMU.1346	sahH	putative thioesterase BacT	bacT	2,39	1,9E-06
SMU.1346	SahH 10 nM	putative thioesterase BacT	bacT	2,61	1,0E-06
SMU.1346	SahH 25 µM	putative thioesterase BacT	bacT	2,80	6,7E-07
SMU.1432c	sahH	putative endoglucanase precursor	-	1,90	8,6E-06
SMU.1432c	SahH 10 nM	putative endoglucanase precursor	-	1,81	1,4E-05
SMU.1432c	SahH 25 µM	putative endoglucanase precursor	-	1,80	1,5E-05
SMU.1476c	sahH	putative GTP-binding protein	-	-1,77	2,9E-05
SMU.1702c	SahH 10 nM	putative phosphatase	-	-1,92	4,1E-02
SMU.1702c	SahH 25 µM	putative phosphatase	-	-1,93	3,9E-02
Mobile and extrachromosomal elements (2, 2, 0)					
SMU.94c	SahH 10 nM	putative putative transposase	-	1,79	2,4E-03
SMU.1808c	sahH	putative integrase fragment	-	2,50	4,9E-02
SMU.1808c	SahH 10 nM	putative integrase fragment	-	2,61	4,1E-02
SMU.1888	sahH	putative transposase	-	1,73	4,0E-03
Phenylalanine, tyrosine and tryptophan biosynthesis (2, 2, 2)					
SMU.1836	sahH	phospho-2-dehydro-3-deoxyheptonate aldolase	aroG	-3,39	2,6E-04
SMU.1836	SahH 10 nM	phospho-2-dehydro-3-deoxyheptonate aldolase	aroG	-3,58	2,0E-04
SMU.1836	SahH 25 µM	phospho-2-dehydro-3-deoxyheptonate aldolase	aroG	-2,96	5,0E-04
SMU.1837	sahH	phospho-2-dehydro-3-deoxyheptonate aldolase	aroH	-36,51	2,9E-04
SMU.1837	SahH 10 nM	phospho-2-dehydro-3-deoxyheptonate aldolase	aroH	-41,39	2,4E-04
SMU.1837	SahH 25 µM	phospho-2-dehydro-3-deoxyheptonate aldolase	aroH	-22,33	6,6E-04
Phosphotransferase system (PTS) (9, 9, 6)					
SMU.114	sahH	putative PTS system, fructose-specific IIBC component	-	1,99	1,5E-02
SMU.114	SahH 10 nM	putative PTS system, fructose-specific IIBC component	-	1,97	1,6E-02
SMU.1185	sahH	PTS system, mannitol-specific enzyme IIBC component	mtlA1	2,15	1,3E-05
SMU.1185	SahH 10 nM	PTS system, mannitol-specific enzyme IIBC component	mtlA1	2,21	1,1E-05

SMU.1596	SahH 10 nM	cellobiose phosphotransferase system IIC component	celD	1,79	3,5E-03
SMU.1877	sahH	putative PTS system, mannose-specific component IIAB	ptnA	1,96	1,8E-08
SMU.1877	SahH 10 nM	putative PTS system, mannose-specific component IIAB	ptnA	1,81	4,0E-08
SMU.1877	SahH 25 µM	putative PTS system, mannose-specific component IIAB	ptnA	1,78	4,8E-08
SMU.1878	sahH	putative PTS system, mannose-specific component IIC	ptnC	2,09	2,6E-07
SMU.1878	SahH 10 nM	putative PTS system, mannose-specific component IIC	ptnC	1,91	5,7E-07
SMU.1878	SahH 25 µM	putative PTS system, mannose-specific component IIC	ptnC	1,83	8,7E-07
SMU.1879	sahH	putative PTS system, mannose-specific component IID	-	2,51	3,2E-08
SMU.1879	SahH 10 nM	putative PTS system, mannose-specific component IID	-	2,31	5,8E-08
SMU.1879	SahH 25 µM	putative PTS system, mannose-specific component IID	-	2,24	7,2E-08
SMU.1957	sahH	putative PTS system, mannose-specific IID component	-	2,38	1,4E-06
SMU.1957	SahH 10 nM	putative PTS system, mannose-specific IID component	-	1,96	6,5E-06
SMU.1957	SahH 25 µM	putative PTS system, mannose-specific IID component	-	1,89	9,2E-06
SMU.1958c	sahH	putative PTS system, mannose-specific IIC component	-	2,64	1,1E-07
SMU.1958c	SahH 10 nM	putative PTS system, mannose-specific IIC component	-	2,15	4,7E-07
SMU.1958c	SahH 25 µM	putative PTS system, mannose-specific IIC component	-	2,11	5,3E-07
SMU.1960c	sahH	putative PTS system, mannose-specific IIB component	-	2,17	3,7E-08
SMU.1960c	SahH 10 nM	putative PTS system, mannose-specific IIB component	-	1,81	2,0E-07
SMU.1960c	SahH 25 µM	putative PTS system, mannose-specific IIB component	-	1,76	2,7E-07
SMU.1961c	sahH	putative PTS system, sugar-specific enzyme IIA component	-	1,71	4,4E-07
Pyruvate metabolism (1,1,1)					
SMU.137	sahH	malate dehydrogenase	mleS	-2,02	3,8E-07
SMU.137	SahH 10 nM	malate dehydrogenase	mleS	-2,11	2,6E-07
SMU.137	SahH 25 µM	malate dehydrogenase	mleS	-2,07	3,1E-07
Starch and sucrose metabolism (4, 2, 1)					
SMU.78	sahH	fructan hydrolase; exo-beta-D-fructosidase; fructanase, FruA	fruA	1,73	3,8E-08
SMU.79	sahH	fructan hydrolase; exo-beta-D-fructosidase; FruB	fruB	1,90	6,9E-05
SMU.910	sahH	glucosyltransferase-S	gtfD	1,88	5,8E-06
SMU.910	SahH 10 nM	glucosyltransferase-S	gtfD	1,77	1,1E-05
SMU.910	SahH 25 µM	glucosyltransferase-S	gtfD	1,79	9,7E-06
SMU.1434c	sahH	putative glycosyltransferase	-	1,70	1,1E-05
SMU.1434c	SahH 10 nM	putative glycosyltransferase	-	1,72	9,6E-06
Transcription (8, 5, 7)					
SMU.144c	sahH	putative transcriptional regulator	-	-1,84	1,0E-06
SMU.144c	SahH 10 nM	putative transcriptional regulator	-	-1,71	2,2E-06
SMU.144c	SahH 25 µM	putative transcriptional regulator	-	-1,81	1,2E-06
SMU.424	sahH	negative transcriptional regulator, CopY	copY	-2,34	1,8E-07
SMU.424	SahH 10 nM	negative transcriptional regulator, CopY	copY	-2,33	1,9E-07
SMU.424	SahH 25 µM	negative transcriptional regulator, CopY	copY	-2,29	2,1E-07
SMU.632	sahH	putative transcriptional regulator	-	-1,80	2,5E-05
SMU.632	SahH 25 µM	putative transcriptional regulator	-	-1,74	3,6E-05
SMU.885	sahH	galactose operon repressor GalR	galR	-1,81	1,8E-07
SMU.885	SahH 10 nM	galactose operon repressor GalR	galR	-1,73	2,9E-07
SMU.885	SahH 25 µM	galactose operon repressor GalR	galR	-1,75	2,5E-07

SMU.1566	sahH	putative maltose operon transcriptional repressor	malR	-1,79	4,5E-06
SMU.1566	SahH 25 µM	putative maltose operon transcriptional repressor	malR	-1,71	7,2E-06
SMU.1647c	sahH	putative transcriptional regulator	-	-1,70	1,5E-04
SMU.1647c	SahH 25 µM	putative transcriptional regulator	-	-1,78	9,6E-05
SMU.1657c	sahH	putative nitrogen regulatory protein PII	-	-1,87	1,7E-04
SMU.1657c	SahH 10 nM	putative nitrogen regulatory protein PII	-	-1,70	4,5E-04
SMU.2027	sahH	putative transcriptional regulator	-	-1,87	7,6E-06
SMU.2027	SahH 10 nM	putative transcriptional regulator	-	-1,97	4,6E-06
SMU.2027	SahH 25 µM	putative transcriptional regulator	-	-1,92	6,0E-06
Translation (22, 22, 21)					
SMU.120	SahH 10 nM	50S ribosomal protein L28	rpmB	1,97	3,8E-05
SMU.120	SahH 25 µM	50S ribosomal protein L28	rpmB	1,93	4,5E-05
SMU.170	sahH	30S ribosomal protein S9	rpsI	1,84	1,7E-07
SMU.170	SahH 10 nM	30S ribosomal protein S9	rpsI	1,87	1,5E-07
SMU.170	SahH 25 µM	30S ribosomal protein S9	rpsI	1,88	1,4E-07
SMU.699	sahH	50S ribosomal protein L20	rplT	1,71	2,0E-06
SMU.699	SahH 10 nM	50S ribosomal protein L20	rplT	1,70	2,1E-06
SMU.960	sahH	50S ribosomal protein L7/L12	rplL	1,81	5,0E-07
SMU.960	SahH 10 nM	50S ribosomal protein L7/L12	rplL	1,84	4,3E-07
SMU.960	SahH 25 µM	50S ribosomal protein L7/L12	rplL	1,88	3,5E-07
SMU.1626	sahH	50S ribosomal protein L1	rplA	1,85	5,0E-08
SMU.1626	SahH 10 nM	50S ribosomal protein L1	rplA	1,84	5,4E-08
SMU.1626	SahH 25 µM	50S ribosomal protein L1	rplA	1,85	5,1E-08
SMU.2007	sahH	50S ribosomal protein L15	rplO	2,30	6,5E-09
SMU.2007	SahH 10 nM	50S ribosomal protein L15	rplO	2,12	1,2E-08
SMU.2007	SahH 25 µM	50S ribosomal protein L15	rplO	2,20	9,2E-09
SMU.2008	sahH	50S ribosomal protein L30	rpmD	2,45	2,5E-09
SMU.2008	SahH 10 nM	50S ribosomal protein L30	rpmD	2,27	4,3E-09
SMU.2008	SahH 25 µM	50S ribosomal protein L30	rpmD	2,29	4,1E-09
SMU.2009	sahH	30S ribosomal protein S5	rpsE	2,35	1,6E-08
SMU.2009	SahH 10 nM	30S ribosomal protein S5	rpsE	2,14	3,4E-08
SMU.2009	SahH 25 µM	30S ribosomal protein S5	rpsE	2,29	1,9E-08
SMU.2010	sahH	50S ribosomal protein L18	rplR	2,55	1,1E-08
SMU.2010	SahH 10 nM	50S ribosomal protein L18	rplR	2,40	1,7E-08
SMU.2010	SahH 25 µM	50S ribosomal protein L18	rplR	2,52	1,2E-08
SMU.2011	sahH	50S ribosomal protein L6	rplF	2,40	1,1E-08
SMU.2011	SahH 10 nM	50S ribosomal protein L6	rplF	2,13	2,7E-08
SMU.2011	SahH 25 µM	50S ribosomal protein L6	rplF	2,25	1,8E-08
SMU.2012	sahH	30S ribosomal protein S8	rpsH	2,57	1,8E-08
SMU.2012	SahH 10 nM	30S ribosomal protein S8	rpsH	2,35	3,4E-08
SMU.2012	SahH 25 µM	30S ribosomal protein S8	rpsH	2,43	2,7E-08
SMU.2014	sahH	30S ribosomal protein S14	rpsN	2,44	1,2E-08
SMU.2014	SahH 10 nM	30S ribosomal protein S14	rpsN	2,26	2,2E-08
SMU.2014	SahH 25 µM	30S ribosomal protein S14	rpsN	2,25	2,3E-08
SMU.2015	sahH	50S ribosomal protein L5	rplE	2,39	8,4E-09

SMU.2015	SahH 10 nM	50S ribosomal protein L5	rplE	2,13	2,0E-08
SMU.2015	SahH 25 µM	50S ribosomal protein L5	rplE	2,24	1,3E-08
SMU.2016	sahH	50S ribosomal protein L24	rplX	2,53	8,6E-09
SMU.2016	SahH 10 nM	50S ribosomal protein L24	rplX	2,22	2,2E-08
SMU.2016	SahH 25 µM	50S ribosomal protein L24	rplX	2,27	1,9E-08
SMU.2017	sahH	50S ribosomal protein L14	rplN	2,36	8,2E-09
SMU.2017	SahH 10 nM	50S ribosomal protein L14	rplN	2,07	2,3E-08
SMU.2017	SahH 25 µM	50S ribosomal protein L14	rplN	2,14	1,7E-08
SMU.2018	sahH	30S ribosomal protein S17	rpsQ	2,44	9,5E-09
SMU.2018	SahH 10 nM	30S ribosomal protein S17	rpsQ	2,20	2,0E-08
SMU.2018	SahH 25 µM	30S ribosomal protein S17	rpsQ	2,24	1,7E-08
SMU.2019	sahH	50S ribosomal protein L29	rpmC	2,41	2,1E-08
SMU.2019	SahH 10 nM	50S ribosomal protein L29	rpmC	2,18	4,3E-08
SMU.2019	SahH 25 µM	50S ribosomal protein L29	rpmC	2,27	3,2E-08
SMU.2020	sahH	50S ribosomal protein L16	rplP	2,26	2,0E-08
SMU.2020	SahH 10 nM	50S ribosomal protein L16	rplP	2,01	5,1E-08
SMU.2020	SahH 25 µM	50S ribosomal protein L16	rplP	2,09	3,7E-08
SMU.2022	sahH	50S ribosomal protein L22	rplV	2,22	1,9E-08
SMU.2022	SahH 10 nM	50S ribosomal protein L22	rplV	2,05	3,7E-08
SMU.2022	SahH 25 µM	50S ribosomal protein L22	rplV	2,07	3,3E-08
SMU.2023c	sahH	30S ribosomal protein S19	-	2,16	2,5E-08
SMU.2023c	SahH 10 nM	30S ribosomal protein S19	-	1,94	6,3E-08
SMU.2023c	SahH 25 µM	30S ribosomal protein S19	-	2,04	4,0E-08
SMU.2031	sahH	elongation factor Ts	tsf	2,04	1,2E-07
SMU.2031	SahH 10 nM	elongation factor Ts	tsf	2,06	1,1E-07
SMU.2031	SahH 25 µM	elongation factor Ts	tsf	1,99	1,4E-07
SMU.2135c	sahH	30S ribosomal protein S4	rpsD	1,81	3,3E-07
SMU.2135c	SahH 10 nM	30S ribosomal protein S4	rpsD	1,84	2,8E-07
SMU.2135c	SahH 25 µM	30S ribosomal protein S4	rpsD	1,84	2,9E-07
SMU.2143c	sahH	tRNA-specific 2-thiouridylase MnmA	mnmA	-1,72	1,2E-07
Transporter (2, 4, 1)					
SMU.138	sahH	putative malate permease	-	-1,96	4,2E-06
SMU.138	SahH 10 nM	putative malate permease	-	-2,14	2,0E-06
SMU.138	SahH 25 µM	putative malate permease	-	-2,05	2,8E-06
SMU.1013c	SahH 10 nM	putative Mg2+/citrate transporter	-	1,77	4,8E-04
SMU.1658	sahH	putative ammonium transporter, NrgA protein	nrgA	-2,17	1,3E-04
SMU.1658	SahH 10 nM	putative ammonium transporter, NrgA protein	nrgA	-1,92	3,6E-04
SMU.2109	SahH 10 nM	putative MDR permease; multidrug efflux pump	-	1,76	1,5E-02

8.4 Supplementary table 4

Supplementary table S4

Confirmation of microarray data with quantitative real time PCR.

Gene	*ΔluxS₂*		*ΔluxS₂*pDLluxS		*ΔluxS₂*SahH		*ΔluxS₂*SahH+ 10 nM AI-2		*ΔluxS₂*SahH+ 25 µM AI-2	
	Micro-array	qPCR	Micro-array	qPCR	Micro-array	qPCR	Micro-array	qPCR	Micro-array	qPCR
SMU.137	-1,31	-1,07±0,15	-5,57	-3,62±0,02	-2,02	-1,34±0,08	-2,11	-1,29±0,03	-2,07	-1,56±0,01
SMU.150	1,0	1,0±0,05	1,04	-1,05±0,03	1,49	1,72±0,11	1,66	1,66±0,24	1,56	1,54±0,23
SMU.1004	-1,52	-1,05±0,28	-2,23	-2,06±0,05	1,35	1,88±0,06	1,41	1,60±0,2	1,65	1,34±0,19
SMU.1005	-1,56	-1,13±0,13	-2,92	-1,26±0,02	1,29	2,15±0,11	1,41	1,68±0,09	1,34	1,62±0,12
SMU.1895c	-1,01	-1,02±0,13	1,48	1,35±0,08	2,2	1,83±0,11	2,06	1,68±0,14	2,13	1,64±0,09
SMU.1957	1,02	1,10±0,11	2,41	2,74±0,03	2,38	2,76±0,09	1,96	2,25±0,21	1,89	2,0±0,17

Data represents the mean and standard deviation of both biological samples measured in duplicate and are presented as fold change compared to the expression of the wildtype.

8.5 Supplementary table 5

Supplementary table S5.

Genes, that were significant changed under at least one condition. Significant changes (fold change > ±1.8; P-value < 0.05) are marked in bold.

Locus Tag SMU.#	Description	Gene symbol	Fold Ind. mixed	P-Value Induced mixed	Fold GFP-	P-Value GFP-	Fold GFP+	P-Value GFP+	Fold GFP+/ GFP-	P-value GFP+ / GFP-
Amino acid biosynthesis										
262	putrescine carbamoyltransferase		0.8	3.71E-01	**0.5**	**3.62E-02**	**0.4**	**1.53E-02**	0.70	7.58E-02
264	agmatine deiminase		0.7	2.86E-01	**0.5**	**3.59E-02**	0.5	6.00E-02	0.96	8.54E-01
265	carbamate kinase		0.8	3.91E-01	**0.5**	**2.44E-02**	0.6	8.30E-02	1.02	9.10E-01
663	N-acetyl-gamma-glutamyl-phosphate reductase		0.8	3.56E-01	**0.5**	**3.27E-02**	**0.5**	**4.51E-02**	0.96	8.30E-01
1044c	putative pseudouridylate synthase		1.2	2.25E-01	1.1	6.05E-01	**1.8**	**9.56E-03**	1.54	8.87E-02
Biosynthesis of cofactors, prosthetic groups, and carriers										
353	hypothetical protein	-	**3.0**	**2.09E-02**	1.7	1.61E-01	**7.1**	**2.19E-03**	**3.11**	**4.89E-02**
838	glutathione reductase	*gshR*	**2.0**	**3.38E-02**	1.6	1.15E-01	**2.2**	**2.16E-02**	1.18	6.41E-01
Purines, pyrimidines, nucleosides and nucleotides										
325	deoxyuridine 5'-triphosphate nucleotidohydrolase		1.3	1.03E-01	1.1	4.37E-01	**2.0**	**3.84E-03**	1.52	**4.85E-02**
Protein synthesis										
500	putative ribosome-associated protein	-	**3.4**	**3.61E-04**	**2.3**	**1.75E-03**	**5.3**	**8.69E-05**	**1.96**	**2.65E-02**
Protein Fate										
645	putative oligopeptidase	*pepB*	**3.7**	**4.79E-03**	**2.2**	**3.20E-02**	**11.0**	**3.49E-04**	**3.69**	**1.69E-02**
665	putative oligopeptidase		0.8	4.16E-01	**0.5**	**3.06E-02**	0.6	1.25E-01	1.12	6.15E-01
Energy metabolism										
352	ribulose-phosphate 3-epimerase	-	**3.0**	**1.55E-02**	1.7	1.52E-01	**7.2**	**1.48E-03**	**3.34**	**2.95E-02**
772	putative glucan-binding protein D; BglB-like protein	*gbpD*	**2.4**	**2.42E-02**	1.4	2.46E-01	**7.3**	**9.35E-04**	**4.07**	**1.57E-02**

1396	glucan-binding protein C, GbpC		1.8	**2.35E-02**	**2.0**	**1.23E-02**	1.3	1.86E-01	0.61	7.57E-02
1978	putative acetate kinase	*ackA*	**6.6**	**5.64E-04**	**2.8**	**6.75E-03**	**14.9**	**1.13E-04**	**4.76**	**7.28E-03**
2115	putative short-chain dehydrogenase		0.8	5.61E-01	0.6	1.33E-01	**0.5**	**4.76E-02**	0.71	3.11E-01
Transport and binding proteins										
28	putative ATP-binding protein		0.8	1.96E-01	0.6	**2.69E-02**	**0.5**	**8.94E-03**	0.74	2.04E-01
109	permease (efflux protein)		1.1	4.16E-01	1.1	5.06E-01	**1.9**	**9.59E-04**	1.73	**8.21E-03**
263	putative amino acid antiporter		0.8	2.05E-01	**0.5**	**1.31E-02**	**0.5**	**1.13E-02**	0.74	1.84E-01
426	copper-transporting ATPase; P-type ATPase	*copA*	**2.0**	**7.04E-03**	**1.9**	**8.58E-03**	**2.3**	**3.34E-03**	1.10	6.29E-01
427	putative copper chaperone	*copZ*	**2.0**	**8.12E-04**	**1.9**	**9.07E-04**	**2.3**	**3.34E-04**	1.11	4.41E-01
470	putative copper chaperone		1.3	**2.58E-02**	1.5	**9.44E-03**	**1.8**	**1.61E-03**	1.20	2.64E-01
654	putative ABC transporter, ATP-binding protein MutF		0.7	2.11E-01	**0.4**	**1.61E-02**	**0.5**	**2.65E-02**	0.91	5.64E-01
655	putative MutE		0.7	5.46E-01	**0.2**	**4.38E-02**	0.3	5.73E-02	1.16	7.16E-01
656	putative MutE		0.7	1.91E-01	**0.4**	**1.67E-02**	**0.4**	**3.39E-02**	1.10	5.82E-01
657	putative MutG		0.6	2.04E-01	**0.2**	**1.17E-02**	**0.3**	**1.98E-02**	0.99	9.40E-01
1148	putative transporter, ATP-binding protein; bacteriocin immunity protein		0.7	5.02E-01	**0.2**	**3.28E-02**	0.3	8.44E-02	1.48	1.66E-01
1149	transporter		0.7	3.98E-01	**0.3**	**2.20E-02**	0.5	1.27E-01	1.42	1.34E-01
1150	transporter		0.6	2.88E-01	**0.3**	**1.41E-02**	0.4	5.83E-02	1.36	9.93E-02
1605	putative MDR permease; transmembrane efflux protein		0.7	7.68E-02	**0.5**	**6.04E-03**	**0.6**	**9.56E-03**	0.98	8.98E-01
1897	putative ABC transporter, ATP-binding protein		1.6	**1.65E-02**	1.6	**1.44E-02**	**2.7**	**8.36E-04**	1.61	8.23E-02
1963c	putative sugar-binding periplasmic protein		1.4	3.60E-01	1.0	9.16E-01	**2.5**	**3.64E-02**	2.07	1.46E-01
1966c	putative periplasmic sugar-binding protein		2.0	9.13E-02	1.6	2.03E-01	**4.4**	**7.70E-03**	2.37	1.33E-01
2057c	putative cadmium-transporting ATPase; P-type ATPase		1.8	8.66E-02	**2.1**	**4.16E-02**	**2.1**	**4.67E-02**	0.90	7.63E-01
Regulatory functions										
61	putative transcriptional regulator		1.4	**1.70E-02**	1.1	2.27E-01	**1.8**	**1.37E-03**	1.40	**4.92E-02**
65	putative protein tyrosine-phosphatase		1.5	2.43E-01	1.0	9.99E-01	**3.4**	**1.26E-02**	2.58	5.99E-02
80	heat-inducible transcription repressor		1.7	**4.57E-02**	1.6	**4.92E-02**	**2.1**	**1.28E-02**	1.08	6.89E-01
134	TetR/AcrR family transcriptional regulator		1.3	2.87E-01	**1.9**	**3.88E-02**	1.8	5.23E-02	0.95	9.01E-01
168	putative transcriptional regulator		1.6	**4.14E-03**	1.4	**1.52E-02**	**2.6**	**1.51E-04**	1.69	**8.93E-03**
292	putative transcriptional regulator		0.7	2.66E-01	**0.5**	**4.94E-02**	0.6	9.64E-02	1.01	9.82E-01
356	pur operon repressor		1.6	**4.13E-02**	1.3	2.39E-01	**2.5**	**4.03E-03**	1.63	1.07E-01
424	negative transcriptional regulator, CopY	*copY*	**2.0**	**3.36E-03**	**2.0**	**3.56E-03**	**2.3**	**1.51E-03**	1.12	4.78E-01
507	DeoR family transcriptional regulator		1.6	**2.98E-02**	1.2	3.38E-01	**3.1**	**1.06E-03**	**2.39**	**9.93E-03**
664	putative competence protein/transcription factor		0.7	1.57E-01	**0.5**	**1.92E-02**	0.6	**3.31E-02**	0.97	8.97E-01
1193	putative transcriptional regulator		**1.7**	**1.63E-02**	1.6	**3.44E-02**	**1.8**	**1.19E-02**	1.10	6.52E-01
1287	putative transcriptional regulator	-	**1.8**	**1.71E-02**	**2.0**	**1.09E-02**	1.6	**4.09E-02**	0.82	3.94E-01
1599	putative transcriptional regulator; possible antiterminator		0.7	5.85E-01	0.3	7.76E-02	**0.2**	**4.72E-02**	0.68	2.61E-01
1977c	putative transcriptional regulator		1.4	**4.90E-02**	1.2	1.62E-01	**2.0**	**2.63E-03**	1.35	1.15E-01
1997	putative ComX1, transcriptional regulator of competence-specific genes	*comX1*	**3.6**	**2.58E-02**	2.0	1.40E-01	**13.8**	**1.50E-03**	**4.96**	**2.48E-02**
1997	qPCR of *comX1* 1. Biological sample	*comX1*	4.09		1.93		6.07			
1997	qPCR of *comX1* 2. Biological sample	*comX1*	7.26		2.68		9.48			
2084c	transcriptional regulator Spx	*spxB*	**1.9**	**7.41E-03**	1.3	1.44E-01	**2.8**	**9.48E-04**	**1.95**	**1.24E-02**

Signal transduction										
927	putative response regulator	-	**2.5**	**5.52E-03**	**2.2**	**8.57E-03**	**3.7**	**1.20E-03**	1.38	2.70E-01
928	putative histidine kinase	-	**2.3**	**5.61E-03**	**2.1**	**8.88E-03**	**3.6**	**1.04E-03**	1.42	2.11E-01
1596	cellobiose phosphotransferase system IIC component		0.7	4.03E-01	**0.4**	**4.44E-02**	**0.4**	**4.49E-02**	0.98	9.44E-01
1916	histidine kinase of the competence regulon, ComD	*comD*	**3.9**	**9.95E-04**	**2.6**	**4.45E-03**	**7.0**	**2.13E-04**	**2.09**	**2.36E-02**
1917	response regulator of the competence regulon, ComE;	*comE*	**3.9**	**4.23E-04**	**2.5**	**2.16E-03**	**7.3**	**7.47E-05**	**2.31**	**7.37E-03**
1917	qPCR of *comE* 1. Biological sample	*comE*	6.51		2.66		11.82			
1917	qPCR of *comE* 2. Biological sample	*comE*	2.97		1.38		6.2			
1964c	putative response regulator		1.5	2.94E-01	1.1	8.21E-01	**2.6**	**3.10E-02**	2.15	1.56E-01
1965c	putative histidine kinase		1.8	1.58E-01	1.3	4.92E-01	**3.5**	**1.77E-02**	2.27	1.29E-01
Transformasome										
498	putative late competence protein	*comF*	**9.4**	**3.86E-04**	**3.6**	**4.25E-03**	**31.8**	**5.38E-05**	**6.93**	**2.28E-03**
499	putative late competence protein	-	**12.6**	**3.65E-04**	**4.8**	**2.88E-03**	**48.0**	**5.36E-05**	**8.35**	**8.07E-03**
539c	signal peptidase type IV	-	**2.8**	**4.14E-03**	**1.8**	**3.91E-02**	**8.7**	**1.75E-04**	**4.32**	**3.73E-03**
625	putative competence protein	*comEA*	**20.3**	**2.10E-04**	**7.1**	**1.38E-03**	**70.9**	**4.32E-05**	**8.26**	**4.83E-03**
626	putative competence protein	-	**18.6**	**1.64E-04**	**6.6**	**1.12E-03**	**55.2**	**3.88E-05**	**6.63**	**5.23E-03**
1980c	hypothetical protein	-	**37.5**	**1.92E-05**	**11.8**	**1.11E-04**	**112.0**	**5.66E-06**	**8.30**	**1.50E-03**
1981c	hypothetical protein	-	**41.4**	**1.52E-05**	**13.1**	**8.33E-05**	**134.9**	**4.25E-06**	**8.47**	**7.13E-04**
1982c	hypothetical protein	-	**35.2**	**1.53E-05**	**11.5**	**8.57E-05**	**116.8**	**3.99E-06**	**8.52**	**6.48E-04**
1983	putative competence protein ComYD	*comYD*	**23.5**	**1.14E-04**	**7.5**	**8.42E-04**	**74.4**	**2.75E-05**	**8.08**	**3.90E-03**
1984	putative competence protein ComYC	*comYC*	**40.3**	**1.58E-05**	**12.2**	**9.46E-05**	**140.4**	**4.12E-06**	**9.17**	**5.54E-04**
1985	ABC transporter ComYB	*comYB*	**30.6**	**2.92E-05**	**9.5**	**1.97E-04**	**91.4**	**8.13E-06**	**7.93**	**5.71E-04**
1987	putative ABC transporter, ATP-binding protein ComYA; late competence gene	*comYA*	**44.8**	**1.51E-05**	**13.3**	**8.81E-05**	**164.4**	**3.86E-06**	**9.63**	**7.10E-04**
1987	qPCR of *comYA* 1. Biological sample	*comYA*	69.64		41.29		119.31			
1987	qPCR of *comYA* 2. Biological sample	*comYA*	104.42		48.41		234.57			
DNA Processing										
64	Holliday junction DNA helicase RuvB		1.6	2.13E-01	1.0	9.49E-01	**3.7**	**9.86E-03**	**2.72**	**4.81E-02**
354	hypothetical protein DNA repair protein RmuC	-	**3.2**	**2.01E-02**	1.7	1.62E-01	**7.7**	**2.12E-03**	**3.37**	**4.75E-02**
505	putative adenine-specific DNA methylase	-	**6.0**	**8.31E-03**	2.4	8.96E-02	**16.6**	**1.31E-03**	**5.45**	**3.11E-02**
506	putative type II restriction endonuclease	-	**3.6**	**2.71E-02**	1.7	2.64E-01	**9.5**	**3.23E-03**	**4.31**	**4.40E-02**
644	putative competence protein/transcription factor	-	**5.9**	**3.17E-03**	**2.8**	**2.53E-02**	**20.1**	**3.29E-04**	**5.33**	**2.11E-02**
647	putative methyltransferase		1.6	**4.48E-03**	1.3	5.69E-02	**2.6**	**2.59E-04**	**1.83**	**1.19E-02**
1001	putative DNA processing Smf protein	*smf*	**12.7**	**5.55E-04**	**4.8**	**4.45E-03**	**45.2**	**8.97E-05**	**7.54**	**5.79E-03**
1002	DNA topoisomerase I	*topA*	**3.2**	**3.67E-02**	1.7	2.43E-01	**7.9**	**4.18E-03**	3.43	7.96E-02
1055	DNA repair protein RadC	*radC*	**6.4**	**1.54E-03**	**2.7**	**1.84E-02**	**20.8**	**1.75E-04**	**5.49**	**8.01E-03**
1259	restriction endonuclease		0.8	5.20E-01	**0.3**	**3.84E-02**	0.4	5.11E-02	0.82	3.72E-01
1967	single-stranded DNA-binding protein	*ssb2*	**28.9**	**3.79E-05**	**9.2**	**2.53E-04**	**105.8**	**8.45E-06**	**9.47**	**1.65E-03**
2085	recombinase A	*recA*	**3.2**	**2.27E-03**	1.7	**3.87E-02**	**6.6**	**2.66E-04**	**3.78**	**3.18E-03**
2086	competence damage-inducible protein A	*cinA*	**4.9**	**5.08E-03**	2.3	5.41E-02	**13.1**	**6.56E-04**	**5.37**	**1.36E-02**
Mobile and extrachromosomal element functions										
93c	putative putative transposase		0.7	2.91E-01	**0.4**	**2.54E-02**	**0.4**	**2.86E-02**	0.92	6.34E-01
94c	putative putative transposase		0.8	2.26E-01	**0.5**	**1.49E-02**	**0.5**	**2.14E-02**	0.98	8.58E-01

436c	putative transposase, ISSMU1		0.6	1.44E-01	**0.4**	**1.58E-02**	**0.4**	**1.30E-02**	0.88	7.50E-01
565c	putative transposase, ISSMU1		0.6	1.94E-01	**0.4**	**1.95E-02**	**0.3**	**1.68E-02**	0.89	7.94E-01
1031	putative transposon excisionase; Tn916 ORF1-like		0.7	6.20E-01	0.2	6.18E-02	**0.2**	**4.96E-02**	0.97	9.11E-01
1356c	putative putative transposase		1.6	8.14E-02	**2.0**	**2.42E-02**	1.5	1.37E-01	0.76	3.53E-01
1812	putative transposase, ISSMU2		0.7	1.08E-01	**0.5**	**9.30E-03**	**0.5**	**4.65E-03**	0.74	1.21E-01
Putative Bacteriocins, Immunity Proteins, Bacteriocin Island										
150	hypothetical protein	*nlmA*	**6.5**	**3.84E-04**	**5.6**	**5.49E-04**	**7.3**	**2.98E-04**	1.10	7.16E-01
150	qPCR of *nlmA* 1. Biological sample	*nlmA*	2.37		4.71		5.71			
150	qPCR of *nlmA* 2. Biological sample	*nlmA*	2.86		4.99		6.0			
151	hypothetical protein	*nlmB*	**6.5**	**5.51E-04**	**5.7**	**7.56E-04**	**7.0**	**4.55E-04**	1.04	8.81E-01
423	hypothetical protein	-	**6.7**	**2.34E-04**	**5.9**	**3.11E-04**	**7.2**	**1.92E-04**	1.04	8.58E-01
925	hypothetical protein	*cipI*	**6.2**	**5.92E-05**	**5.2**	**9.33E-05**	**8.7**	**2.66E-05**	1.38	1.10E-01
925	qPCR of *cipI* 1. Biological sample	*cipI*	17.57		9.37		26.81			
925	qPCR of *cipI* 2. Biological sample	*cipI*	3.24		4.0		6.22			
1892c	hypothetical protein		0.9	6.18E-01	0.5	7.56E-02	**0.5**	**3.52E-02**	0.71	1.82E-01
1902c	hypothetical protein	-	**2.8**	**7.64E-04**	**2.8**	**8.09E-04**	**3.1**	**5.09E-04**	0.96	8.32E-01
1903c	hypothetical protein	-	**4.9**	**1.04E-04**	**4.8**	**1.08E-04**	**5.7**	**6.90E-05**	1.00	9.90E-01
1904c	hypothetical protein	-	**4.5**	**3.74E-04**	**4.3**	**4.19E-04**	**5.0**	**2.73E-04**	0.99	9.47E-01
1905c	putative bacteriocin secretion protein	-	**4.8**	**2.74E-04**	**4.8**	**2.86E-04**	**5.4**	**2.01E-04**	0.96	8.53E-01
1906c	hypothetical protein	-	**6.6**	**1.91E-04**	**6.5**	**2.03E-04**	**6.4**	**2.10E-04**	0.84	5.51E-01
1908c	hypothetical protein	-	**6.4**	**1.91E-04**	**6.1**	**2.11E-04**	**6.4**	**1.90E-04**	0.88	5.48E-01
1909c	hypothetical protein	-	**6.2**	**2.40E-04**	**5.9**	**2.72E-04**	**6.0**	**2.61E-04**	0.88	6.32E-01
1910c	hypothetical protein	-	**6.8**	**2.47E-04**	**6.6**	**2.64E-04**	**6.6**	**2.64E-04**	0.87	6.56E-01
1912c	hypothetical protein	-	**6.1**	**1.52E-04**	**5.6**	**1.86E-04**	**6.1**	**1.53E-04**	0.92	6.98E-01
1913c	putative immunity protein, BLpL-like	-	**5.7**	**2.78E-04**	**5.5**	**3.06E-04**	**5.9**	**2.58E-04**	0.89	6.10E-01
1914c	hypothetical protein	*cipB*	**8.6**	**1.02E-04**	**8.6**	**1.02E-04**	**8.2**	**1.12E-04**	0.78	4.58E-01
1914c	qPCR of *cipB* 1. Biological sample	*cipB*	4.53		3.31		3.95			
1914c	qPCR of *cipB* 2. Biological sample	*cipB*	3.59		4.09		3.68			
Hypotethical proteins										
41	hypothetical protein		0.7	**4.70E-02**	**0.5**	**6.99E-03**	0.6	**2.53E-02**	1.52	3.74E-01
66	hypothetical protein		1.3	1.99E-01	1.0	9.78E-01	**2.1**	**1.20E-02**	1.66	7.80E-02
108	hypothetical protein		1.2	1.19E-01	1.2	1.98E-01	**2.5**	**4.69E-04**	**2.31**	**1.05E-02**
152	hypothetical protein	-	**4.9**	**9.18E-04**	**4.3**	**1.36E-03**	**6.1**	**5.12E-04**	1.22	5.05E-01
153	hypothetical protein	-	**5.4**	**1.94E-04**	**4.6**	**3.21E-04**	**6.8**	**1.11E-04**	1.22	3.58E-01
166	hypothetical protein		1.4	**5.87E-03**	1.3	**1.85E-02**	**2.3**	**1.57E-04**	1.61	**1.03E-02**
167	hypothetical protein		1.3	**3.29E-02**	1.1	2.16E-01	**2.1**	**6.91E-04**	1.65	**1.25E-02**
219	hypothetical protein		1.6	5.43E-02	**2.0**	**1.60E-02**	1.7	**3.83E-02**	0.87	6.41E-01
276c	hypothetical protein		0.8	6.07E-01	**0.3**	**3.86E-02**	0.5	1.23E-01	1.57	1.40E-01
508	hypothetical protein		1.5	**2.48E-02**	1.2	1.70E-01	**2.7**	**5.84E-04**	**2.04**	**1.06E-02**
566c	hypothetical protein		0.8	3.77E-01	0.5	5.26E-02	**0.4**	**4.29E-02**	0.91	8.30E-01
627	hypothetical protein	-	**2.0**	**3.46E-02**	1.3	3.58E-01	**3.6**	**3.58E-03**	2.32	6.50E-02
658	hypothetical protein		0.6	1.36E-01	**0.3**	**6.27E-03**	**0.4**	**1.05E-02**	1.09	5.53E-01
739c	hypothetical protein		0.7	2.71E-01	**0.5**	**3.56E-02**	**0.5**	**2.96E-02**	0.92	6.18E-01
750c	hypothetical protein		0.7	4.73E-01	0.3	5.35E-02	**0.2**	**3.30E-02**	1.03	9.40E-01
753	hypothetical protein		1.3	1.52E-01	1.0	9.93E-01	**2.1**	**4.86E-03**	1.77	**2.19E-02**
769	hypothetical protein	-	**12.2**	**3.59E-04**	**4.7**	**2.90E-03**	**36.0**	**7.11E-05**	**6.44**	**5.75E-03**
836	hypothetical protein	-	**9.9**	**7.66E-04**	**3.8**	**7.22E-03**	**34.4**	**1.11E-04**	**6.61**	**3.26E-03**
1000	hypothetical protein		1.5	1.38E-01	**1.9**	**3.82E-02**	**1.8**	**4.29E-02**	1.00	9.93E-01

1029	hypothetical protein		0.6	4.14E-01	**0.2**	**2.83E-02**	**0.1**	**2.06E-02**	0.89	7.34E-01
1042	hypothetical protein		1.6	9.22E-02	**1.9**	**3.73E-02**	1.7	7.10E-02	0.89	7.70E-01
1070c	hypothetical protein		1.6	1.56E-01	1.9	7.07E-02	**2.3**	**3.69E-02**	1.16	7.53E-01
1155	hypothetical protein		0.7	1.05E-01	**0.4**	**6.84E-03**	**0.5**	**2.87E-02**	1.28	4.27E-01
1368	hypothetical protein		0.9	5.29E-01	**0.5**	**3.39E-02**	0.9	5.76E-01	1.46	7.10E-02
1374	hypothetical protein		1.0	7.74E-01	0.9	6.45E-01	**1.9**	**2.18E-03**	**1.87**	**9.81E-03**
1395c	hypothetical protein	-	**2.3**	**3.26E-03**	**3.0**	**1.10E-03**	**2.3**	**3.54E-03**	0.78	3.42E-01
1399	hypothetical protein		0.7	8.29E-02	**0.5**	**8.99E-03**	**0.5**	**1.05E-02**	0.96	8.64E-01
1400c	hypothetical protein	-	**2.1**	**7.21E-03**	1.5	6.45E-02	**3.8**	**6.08E-04**	**2.02**	**3.80E-02**
1411	hypothetical protein		0.7	3.78E-01	**0.4**	**4.33E-02**	**0.3**	**3.45E-02**	0.73	2.01E-01
1575c	hypothetical protein		1.9	5.66E-02	**2.6**	**1.29E-02**	1.8	6.34E-02	0.71	4.06E-01
1597c	hypothetical protein		0.7	5.36E-01	0.2	5.55E-02	**0.2**	**2.95E-02**	0.88	7.60E-01
1884c	hypothetical protein		0.7	1.59E-01	**0.5**	**1.88E-02**	0.6	5.26E-02	1.08	7.32E-01
1975c	hypothetical protein		1.3	7.47E-02	1.2	2.88E-01	**2.0**	**3.27E-03**	1.45	1.00E-01
1976c	hypothetical protein		1.4	**3.91E-02**	1.3	1.20E-01	**2.2**	**1.91E-03**	1.37	1.15E-01
1979c	hypothetical protein	-	**16.3**	**1.38E-04**	**5.7**	**1.13E-03**	**44.9**	**3.35E-05**	**6.83**	**3.61E-03**
2048	hypothetical protein		0.7	1.22E-01	**0.5**	**1.67E-02**	**0.5**	**1.68E-02**	0.89	4.75E-01
2076c	hypothetical protein	-	**2.4**	**7.39E-03**	1.7	**3.80E-02**	**5.1**	**5.08E-04**	**2.65**	**2.36E-02**
2083c	hypothetical protein		1.0	8.89E-01	0.9	5.16E-01	**1.8**	**3.43E-02**	**1.81**	**3.50E-02**
2133c	hypothetical protein		0.7	7.43E-02	**0.5**	**9.41E-03**	0.6	**1.38E-02**	0.97	8.71E-01

8.6 **Supplementary table 6**

Supplementary table S6.

IGR that were significant changed (log2 fold change > 0.85 and P-value < 0.05) under at least one condition are marked in bold.

Genomic Position	**IGR within ORFs (SMU.#)**		**Fold Ind. Mixed**	**P-Value Induced Mixed**	**Fold GFP-**	**P-Value GFP-**	**Fold GFP+**	**P-Value GFP+**	**Fold GFP+/ GFP-**	**P-Value GFP+/ GFP-**
1_193		01	**0.46**	2.03E-01	**0.16**	**1.80E-02**	**0.12**	**1.08E-02**	1.04	9,53E-01
1553_1707	**01**	02	1.53	7.75E-02	**1.81**	**2.88E-02**	1.67	**4.58E-02**	0.92	7,98E-01
16762_16876	**18**	SMUr01	**2.02**	**1.16E-02**	**1.94**	**1.45E-02**	**1.99**	**1.24E-02**	0.85	5,00E-01
18559_18816	**SMUt01**	SMUr02	1.76	**4.30E-03**	**1.85**	**2.99E-03**	**2.12**	**1.29E-03**	1.00	9,98E-01
27471_27558	**23**	24	0.69	2.88E-01	**0.38**	**2.85E-02**	**0.26**	**8.62E-03**	0.79	7,38E-01
30936_31162	**27**	28	0.76	7.56E-02	0.60	**9.66E-03**	**0.46**	**1.73E-03**	0.73	9,29E-02
31745_31866	**28**	29	0.80	7.50E-01	**0.18**	5.21E-02	**0.09**	**1.58E-02**	0.72	3,77E-01
43665_43831	**38c**	39	0.62	**4.66E-02**	**0.42**	**5.10E-03**	**0.45**	**7.00E-03**	1.01	9,62E-01
44342_44517	**39**	40	0.70	6.23E-01	**0.23**	8.46E-02	**0.15**	**3.90E-02**	**0.47**	**2,94E-02**
50816_51038	**47**	48	**0.55**	3.83E-01	**0.17**	**3.80E-02**	**0.11**	**1.91E-02**	0.69	4,54E-01
61249_61630	**60**	61	1.29	1.98E-01	1.06	7.63E-01	**2.62**	**3.08E-03**	**2.29**	**3,46E-02**
62546_63078	**61**	63c	**15.37**	**1.11E-04**	**6.59**	**5.86E-04**	**81.03**	**1.26E-05**	**11.76**	**7,48E-04**
64921_65216	**63c**	64	**2.63**	**3.15E-02**	1.68	1.70E-01	**10.79**	**9.06E-04**	**4.90**	**1,25E-02**
66715_66778	**65**	66	**1.95**	1.12E-01	1.29	4.94E-01	**3.87**	**1.25E-02**	2.69	1,27E-01
68950_69214	**67**	68	1.55	**1.82E-02**	1.25	1.34E-01	**1.82**	**5.47E-03**	1.38	1,40E-01
74855_74926	**73**	74	0.78	2.12E-01	0.62	**4.33E-02**	**0.43**	**5.09E-03**	0.45	2,14E-01
81848_81917	**78**	79	0.70	8.61E-02	**0.52**	**1.18E-02**	**0.51**	**1.01E-02**	0.90	6,57E-01

83478_83651	**79**	80	1.79	**4.47E-02**	1.72	5.47E-02	**2.15**	**1.77E-02**	1.01	9,62E-01
92009_92178	**87**	88c	0.65	1.87E-01	**0.35**	**1.40E-02**	**0.26**	**5.21E-03**	0.57	**3,19E-02**
93037_93253	**88c**	89c	0.58	5.62E-02	**0.29**	**2.90E-03**	**0.29**	**3.02E-03**	0.73	1,90E-01
95862_95979	**92c**	93c	**0.53**	**1.23E-02**	**0.37**	**2.01E-03**	**0.34**	**1.35E-03**	0.86	4,14E-01
96091_96290	**93c**	94c	0.73	8.77E-02	**0.54**	**9.76E-03**	0.60	**1.83E-02**	1.23	5,42E-01
96462_97181	**94c**	96	**0.44**	**4.01E-02**	**0.23**	**4.33E-03**	**0.25**	**5.51E-03**	0.94	8,79E-01
111685_111944	**110**	112c	**0.41**	**4.44E-02**	**0.15**	**2.69E-03**	**0.06**	**5.18E-04**	0.67	5,74E-01
112695_112822	**112c**	113	0.92	9.04E-01	**0.27**	1.07E-01	**0.16**	**4.35E-02**	1.03	9,49E-01
116635_116734	**116**	117c	0.97	9.35E-01	**0.30**	**3.06E-02**	**0.13**	**3.92E-03**	**0.46**	**2,50E-02**
118091_118704	**117c**	118c	0.59	1.34E-01	**0.38**	**2.33E-02**	**0.54**	8.74E-02	1.26	4,65E-01
120677_121102	**119**	120	0.56	5.26E-02	**0.35**	**6.62E-03**	**0.34**	**5.77E-03**	0.91	7,20E-01
127303_127504	**123**	124	0.65	5.35E-02	**0.48**	**8.71E-03**	**0.49**	**9.34E-03**	0.95	8,55E-01
136886_137007	**132**	133c	1.73	2.73E-01	**3.43**	**4.15E-02**	1.28	6.00E-01	0.24	1,31E-01
138403_138552	**133c**	134	0.55	3.07E-01	**0.20**	**2.80E-02**	**0.10**	**8.44E-03**	0.69	4,31E-01
140365_140553	**135**	136c	**0.50**	3.21E-01	**0.15**	**2.96E-02**	**0.10**	**1.51E-02**	0.80	6,92E-01
147132_147294	**141**	143c	0.69	7.85E-02	**0.47**	**7.29E-03**	**0.36**	**2.14E-03**	0.66	9,03E-02
153302_153812	**149**	150	0.79	2.09E-01	0.60	**2.49E-02**	**0.54**	**1.36E-02**	0.84	4,25E-01
154262_154455	**151**	152	**5.29**	**1.58E-03**	**5.04**	**1.80E-03**	**6.32**	**1.02E-03**	1.08	8,24E-01
154876_155367	**152**	153	**4.70**	**6.99E-04**	**4.26**	**9.33E-04**	**6.42**	**3.10E-04**	1.32	3,43E-01
155470_156211	**153**	154	**4.88**	**1.03E-03**	**4.53**	**1.27E-03**	**6.42**	**5.11E-04**	1.21	5,39E-01
165436_165623	**162c**	163c	0.78	**3.11E-02**	0.73	**1.41E-02**	0.68	**7.13E-03**	0.90	4,27E-01
168122_168252	**165**	166	1.78	**2.18E-03**	1.57	**5.87E-03**	**2.43**	**3.26E-04**	1.57	**2,02E-02**
169406_169698	**168**	_R0081	1.64	**6.68E-03**	1.32	5.32E-02	**2.34**	**6.90E-04**	1.61	9,25E-02
171555_171751	**173**	174c	0.76	2.50E-01	**0.45**	**1.46E-02**	**0.48**	**1.94E-02**	0.81	3,68E-01
172839_172925	**175**	176	0.63	8.83E-02	**0.36**	**6.32E-03**	**0.39**	**8.74E-03**	0.92	7,52E-01
179653_179873	**181**	182	1.69	7.64E-02	**2.68**	**9.38E-03**	**1.97**	**3.50E-02**	0.77	4,63E-01
191947_192028	**SMUt33**	191c	0.72	5.35E-01	**0.20**	**2.39E-02**	**0.42**	1.37E-01	1.81	2,64E-01
204465_204698	**207c**	208c	**0.47**	1.78E-01	**0.21**	**2.42E-02**	**0.19**	**1.90E-02**	0.84	8,50E-01
207200_207418	**210c**	211c	0.67	4.18E-01	**0.38**	8.94E-02	**0.30**	**4.75E-02**	1.00	9,96E-01
213705_213754	**222c**	223c	0.94	8.65E-01	**0.40**	**4.48E-02**	**0.49**	9.12E-02	1.26	2,42E-01
213857_213905	**223c**	224c	0.83	7.14E-01	**0.34**	8.05E-02	**0.23**	**3.22E-02**	0.92	7,83E-01
215093_215840	**226c**	227c	0.75	6.81E-01	**0.25**	8.69E-02	**0.16**	**4.02E-02**	0.92	8,42E-01
218822_218986	**229**	231	0.67	5.40E-01	**0.21**	5.04E-02	**0.13**	**2.04E-02**	0.66	3,71E-01
227417_227611	**238c**	239c	0.70	5.21E-01	**0.28**	5.91E-02	**0.16**	**1.77E-02**	0.67	3,04E-01
228203_228504	**239c**	241c	0.67	5.45E-01	**0.22**	6.12E-02	**0.14**	**2.61E-02**	0.73	4,40E-01
250773_250988	**260**	261c	0.80	2.43E-01	0.56	**2.00E-02**	**0.50**	**1.02E-02**	0.82	3,05E-01
251946_252183	**261c**	262	**0.45**	1.51E-01	**0.15**	**1.07E-02**	**0.07**	**2.68E-03**	0.58	4,83E-01
253204_253300	**262**	263	**0.55**	2.63E-01	**0.19**	**1.98E-02**	**0.20**	**2.15E-02**	0.73	5,70E-01
261024_261354	**268**	270	0.74	4.25E-01	**0.40**	**4.90E-02**	**0.34**	**3.02E-02**	0.77	2,39E-01
265999_266179	**275**	276c	0.76	1.05E-01	**0.55**	**8.50E-03**	**0.42**	**1.99E-03**	0.64	1,01E-01
300900_301121	**314**	317	**0.53**	1.62E-01	**0.22**	**1.22E-02**	**0.26**	**1.90E-02**	1.19	5,75E-01
307199_307518	**323**	325	1.26	1.11E-01	1.07	5.84E-01	**1.99**	**2.42E-03**	1.70	**1,70E-02**
315673_315868	**333**	334	0.88	7.80E-01	**0.39**	8.91E-02	**0.24**	**2.63E-02**	0.71	1,91E-01
318785_318878	**335**	336	0.78	7.15E-01	**0.23**	7.33E-02	**0.14**	**3.05E-02**	0.86	6,55E-01
349799_349983	**367**	368c	0.67	**3.63E-02**	**0.48**	**3.59E-03**	**0.51**	**4.90E-03**	0.96	8,02E-01
351899_352359	**369c**	370	0.65	3.15E-01	**0.22**	**1.23E-02**	**0.45**	9.20E-02	2.08	1,17E-01
360684_361057	**381c**	382c	0.67	1.06E-01	**0.43**	**1.01E-02**	**0.41**	**8.52E-03**	0.88	5,07E-01
361913_362046	**382c**	383c	0.78	1.74E-01	0.61	**2.83E-02**	**0.52**	**1.01E-02**	0.78	1,84E-01
363094_363266	**383c**	384	0.79	5.98E-01	**0.52**	1.80E-01	**0.30**	**3.74E-02**	0.73	2,94E-01
367013_367066	**390**	391c	1.68	1.33E-01	**2.63**	**2.22E-02**	**1.97**	6.80E-02	0.76	4,52E-01
368829_368962	**392c**	393	0.77	4.66E-01	**0.37**	**3.32E-02**	**0.41**	**4.59E-02**	0.94	7,19E-01
369898_370155	**394c**	395	**0.48**	2.54E-01	**0.15**	**2.16E-02**	**0.09**	**9.10E-03**	0.79	6,84E-01
373417_373587	**396**	399	**0.42**	1.32E-01	**0.12**	**7.79E-03**	**0.09**	**4.76E-03**	1.09	8,98E-01
375868_376028	**401c**	402	1.55	5.32E-02	1.74	**2.46E-02**	**2.26**	**5.85E-03**	1.25	4,79E-01

383776_383903	**408**	409	**0.40**	1.04E-01	**0.14**	**9.27E-03**	**0.12**	**6.98E-03**	1.34	6,69E-01
396762_397151	**423**	424	**4.99**	**1.24E-03**	**4.62**	**1.53E-03**	**5.70**	**8.79E-04**	1.01	9,69E-01
400037_400276	**427**	428	**1.99**	**6.13E-04**	**1.83**	**1.05E-03**	**2.20**	**3.33E-04**	1.11	4,56E-01
401703_402053	**429c**	431	0.74	2.85E-01	0.57	7.77E-02	**0.37**	**1.19E-02**	0.64	2,02E-01
407087_407738	**436c**	438c	0.71	2.54E-01	**0.49**	**4.93E-02**	**0.44**	**2.99E-02**	0.87	7,20E-01
412041_412320	**438c**	439	0.63	3.75E-01	**0.18**	**1.79E-02**	0.83	7.06E-01	**3.87**	**1,18E-03**
435807_435921	**466**	467	1.59	2.09E-01	**2.53**	**3.67E-02**	**2.06**	7.75E-02	0.84	7,41E-01
438046_438145	**467**	469	**0.35**	**1.88E-02**	**0.18**	**2.74E-03**	**0.12**	**1.11E-03**	1.20	4,48E-01
443688_443831	**474**	475	0.76	3.12E-01	**0.35**	**8.08E-03**	**0.11**	**3.85E-04**	0.61	**3,13E-02**
465066_465398	**495**	496	0.62	4.53E-01	**0.19**	**4.17E-02**	**0.10**	**1.43E-02**	**0.43**	**2,62E-02**
466326_466415	**496**	497c	0.71	5.19E-01	**0.23**	**3.48E-02**	**0.17**	**1.86E-02**	0.89	7,43E-01
467046_467117	**497c**	498	**0.55**	3.14E-01	**0.18**	**2.63E-02**	**0.13**	**1.35E-02**	0.82	6,98E-01
469082_469159	**499**	500	**13.41**	**5.67E-05**	**6.74**	**2.29E-04**	**44.07**	**1.00E-05**	**5.66**	**3,98E-03**
469709_469831	**500**	501	0.78	2.46E-01	**0.52**	**2.00E-02**	0.73	1.63E-01	1.12	5,09E-01
472647_472725	**502**	503c	0.62	3.25E-01	**0.31**	**4.89E-02**	**0.22**	**1.98E-02**	0.86	6,51E-01
476209_476731	**506**	507	**3.54**	**2.40E-02**	1.71	2.25E-01	**8.68**	**3.05E-03**	**3.70**	**4,59E-02**
479448_479521	**509**	510c	0.70	3.42E-01	**0.32**	**2.15E-02**	0.63	2.31E-01	1.37	5,86E-01
480158_480284	**510c**	512c	1.52	5.04E-02	1.68	**2.54E-02**	**1.80**	**1.65E-02**	1.09	7,33E-01
486684_486755	**518**	520	0.76	6.34E-01	**0.28**	7.33E-02	**0.16**	**2.30E-02**	0.76	3,55E-01
538324_538498	**575c**	576	0.68	1.21E-01	**0.40**	**7.72E-03**	**0.41**	**8.42E-03**	0.89	5,38E-01
562325_562430	**603**	604	0.59	3.87E-01	**0.24**	5.08E-02	**0.18**	**2.88E-02**	1.20	6,38E-01
569080_569316	**609**	610	**0.52**	6.31E-02	**0.43**	**2.72E-02**	**0.46**	**3.76E-02**	1.58	4,25E-01
578553_579128	**618**	620	0.66	1.68E-01	**0.43**	**2.20E-02**	**0.52**	5.06E-02	1.17	6,84E-01
581145_581232	**622c**	623c	0.90	8.80E-01	**0.40**	2.09E-01	**0.21**	5.98E-02	0.47	7,33E-02
582169_582306	**623c**	624	0.65	3.49E-01	**0.39**	7.21E-02	**0.23**	**1.80E-02**	0.67	1,87E-01
586102_586286	**626**	627	**7.91**	**2.13E-04**	**3.45**	**2.03E-03**	**20.78**	**3.72E-05**	**5.12**	**3,23E-03**
586914_587080	**627**	628	1.67	**1.77E-02**	1.47	**4.67E-02**	**3.34**	**5.36E-04**	**2.07**	**1,18E-02**
596121_596312	**636**	637c	1.77	1.13E-01	**2.15**	5.11E-02	**2.19**	**4.74E-02**	0.95	8,98E-01
602451_602757	**643**	644	**2.70**	**2.30E-02**	1.26	4.70E-01	**8.21**	**1.17E-03**	**5.02**	**9,66E-03**
605518_605729	**645**	646	**3.38**	**8.51E-03**	**2.07**	5.09E-02	**10.16**	**5.71E-04**	**3.53**	**2,25E-02**
606348_606396	**646**	647	0.88	7.03E-01	**0.41**	**4.33E-02**	**0.37**	**3.20E-02**	1.13	6,39E-01
607105_607165	**647**	648	**2.20**	**1.90E-02**	1.78	5.24E-02	**3.79**	**2.42E-03**	1.98	1,39E-01
617136_617510	**657**	658	0.60	4.09E-01	**0.15**	**2.08E-02**	**0.17**	**2.63E-02**	1.09	8,69E-01
621808_621941	**662**	663	0.62	2.51E-01	**0.32**	**2.77E-02**	**0.17**	**5.24E-03**	0.68	1,38E-01
625083_625217	**665**	666	0.93	7.32E-01	**0.48**	**1.89E-02**	0.82	3.83E-01	1.34	7,37E-02
630587_630840	**669c**	670	0.90	8.42E-01	**0.26**	**4.46E-02**	0.56	2.96E-01	**3.12**	**2,00E-03**
688177_688444	**728**	730	0.65	5.66E-01	**0.19**	6.68E-02	**0.13**	**3.83E-02**	0.66	2,81E-01
694747_695055	**739c**	741	1.42	1.52E-01	**1.80**	**3.69E-02**	1.49	1.10E-01	0.84	5,42E-01
696115_696177	**741**	742	1.58	6.29E-02	**1.87**	**2.27E-02**	1.60	5.86E-02	0.83	4,53E-01
700766_700874	**745**	746c	0.74	4.94E-01	**0.27**	**2.48E-02**	**0.44**	1.02E-01	1.28	7,14E-01
702752_703471	**748**	750c	**0.54**	2.66E-01	**0.14**	**1.12E-02**	**0.23**	**3.26E-02**	1.12	7,50E-01
706503_706589	**752**	753	1.39	6.25E-02	1.00	9.97E-01	**2.22**	**2.58E-03**	**1.88**	**4,22E-02**
706890_707086	**753**	754	1.24	2.25E-01	0.99	9.30E-01	**2.10**	**5.48E-03**	**1.83**	**1,04E-02**
715015_715084	**765**	766	**2.34**	9.05E-02	**2.99**	**4.37E-02**	1.46	3.92E-01	0.50	2,15E-01
716434_716479	**767**	768c	0.72	5.66E-01	**0.21**	**3.64E-02**	**0.11**	**1.09E-02**	0.75	3,90E-01
716798_716930	**768c**	769	**2.75**	**5.65E-03**	**2.46**	**8.87E-03**	**2.37**	**1.04E-02**	1.06	8,51E-01
717144_717332	**769**	770c	**14.14**	**8.95E-04**	**5.15**	**6.66E-03**	**53.81**	**1.45E-04**	**8.61**	**8,82E-03**
718674_719212	**770c**	771c	**4.53**	**4.54E-03**	**3.03**	**1.51E-02**	**17.74**	**2.85E-04**	**4.85**	**3,61E-02**
719348_719788	**771c**	772	**2.86**	**7.79E-03**	1.45	1.77E-01	**8.70**	**3.66E-04**	**4.83**	**5,76E-03**
723845_724248	**773c**	774	0.71	1.07E-01	**0.44**	**6.39E-03**	1.11	5.68E-01	**3.35**	**4,34E-02**
727611_727742	**775c**	776	**0.53**	6.72E-02	**0.18**	**1.82E-03**	**0.07**	**2.82E-04**	0.44	1,06E-01
745624_745833	**799c**	800	1.46	1.28E-01	**2.14**	**1.52E-02**	1.37	1.85E-01	0.69	2,24E-01
755296_755491	**807**	809	**2.42**	1.11E-01	**3.18**	5.39E-02	**3.61**	**3.90E-02**	1.11	8,80E-01
759342_759398	**813**	814	1.67	5.56E-02	**2.08**	**1.68E-02**	**1.92**	**2.52E-02**	0.95	8,74E-01

759864_759970	**814**	815	1.71	6.31E-02	**1.86**	**4.05E-02**	1.70	6.61E-02	0.79	5,23E-01
787261_787378	**836**	837	**13.72**	**2.74E-04**	**5.74**	**1.62E-03**	**42.03**	**5.45E-05**	**5.89**	**1,32E-02**
788222_788353	**837**	838	**5.46**	**7.77E-04**	**2.78**	**6.52E-03**	**17.16**	**7.70E-05**	**4.45**	**1,06E-02**
791769_791891	**840c**	841	0.92	8.09E-01	**0.37**	**3.71E-02**	**0.52**	1.17E-01	1.60	1,83E-01
814285_814686	**864**	865	1.63	**1.33E-02**	1.32	8.03E-02	**1.89**	**4.81E-03**	1.35	1,50E-01
837975_838127	**883**	885	**3.64**	**3.47E-02**	**3.71**	**3.29E-02**	**1.86**	2.20E-01	0.52	2,20E-01
844419_844621	**889**	890	**2.06**	**4.32E-02**	**2.50**	**1.95E-02**	1.60	1.37E-01	0.66	2,64E-01
845165_845295	**890**	891	1.67	5.46E-02	**2.01**	**1.96E-02**	1.48	1.13E-01	0.76	3,30E-01
849881_849953	**893**	895	0.66	3.44E-01	**0.31**	**3.56E-02**	**0.26**	**2.34E-02**	1.08	7,98E-01
878575_878770	**923**	924	**1.94**	**2.86E-02**	**2.59**	**7.54E-03**	**2.08**	**2.00E-02**	0.82	5,52E-01
879864_880090	**925**	926	**5.84**	**2.33E-05**	**5.64**	**2.55E-05**	**9.85**	**7.03E-06**	**1.82**	**1,97E-02**
888406_888611	**936**	937	**0.37**	1.21E-01	**0.18**	**2.46E-02**	**0.14**	**1.57E-02**	1.09	9,03E-01
895307_895513	**943c**	944	0.58	3.84E-01	**0.21**	**4.08E-02**	**0.13**	**1.77E-02**	0.68	4,42E-01
896354_896469	**944**	946	1.66	1.18E-01	**2.07**	**4.36E-02**	1.43	2.40E-01	0.74	4,15E-01
897250_897333	**946**	947	1.33	2.54E-01	0.95	8.32E-01	**0.30**	**3.30E-03**	0.61	1,47E-01
903962_904058	**953c**	954	0.70	4.96E-01	**0.36**	8.71E-02	**0.24**	**3.22E-02**	1.01	9,69E-01
928018_928249	**982**	983	0.56	4.08E-01	**0.18**	**4.40E-02**	**0.15**	**3.42E-02**	0.85	5,87E-01
929156_929339	**983**	984	0.69	4.89E-01	**0.29**	5.28E-02	**0.20**	**2.31E-02**	0.85	6,36E-01
931411_931497	**985**	986c	0.55	2.86E-01	**0.18**	**1.96E-02**	**0.12**	**9.64E-03**	0.91	8,71E-01
938355_938638	**991**	992	**2.15**	1.06E-01	**4.15**	**1.54E-02**	**2.61**	5.73E-02	0.68	4,76E-01
945846_945911	**1000**	1001	**0.28**	**1.63E-02**	**0.17**	**4.34E-03**	**0.14**	**2.82E-03**	0.90	7,53E-01
946755_946848	**1001**	1002	**25.91**	**1.87E-03**	**8.89**	**9.45E-03**	**44.84**	**9.56E-04**	**4.47**	**2,53E-02**
948967_949331	**1002**	1003	**2.54**	**4.62E-02**	1.40	3.74E-01	**5.83**	**4.60E-03**	3.07	6,68E-02
950667_951111	**1003**	1004	**2.41**	**2.84E-02**	1.54	1.88E-01	**5.62**	**2.09E-03**	**2.73**	**4,84E-02**
969385_969626	**1013c**	1014	0.93	8.66E-01	**0.41**	1.03E-01	**0.14**	**7.32E-03**	**0.34**	**2,76E-02**
976692_976837	**1024c**	1025	0.67	3.00E-01	**0.29**	**1.70E-02**	**0.29**	**1.73E-02**	0.62	5,18E-02
977264_977309	**1025**	1026	1.78	**2.20E-02**	**1.93**	**1.35E-02**	1.49	7.20E-02	0.74	3,74E-01
980707_981072	**1030**	1031	**0.35**	8.97E-02	**0.12**	**9.35E-03**	**0.08**	**4.51E-03**	0.81	7,65E-01
989163_989322	**1040c**	1041	0.80	7.17E-01	**0.26**	7.84E-02	**0.21**	**4.87E-02**	0.82	4,03E-01
996212_996313	**1048**	1050	**2.13**	6.85E-02	**2.95**	**2.21E-02**	**1.91**	1.05E-01	0.71	4,59E-01
998766_999060	**1052**	1053	1.51	2.21E-01	**2.25**	**4.24E-02**	1.69	1.37E-01	0.76	5,59E-01
1005238_1005346	**1061**	1062	**0.54**	1.12E-01	**0.24**	**7.09E-03**	**0.18**	**3.51E-03**	0.83	4,15E-01
1008295_1008566	**1063**	1064c	**2.37**	**4.82E-02**	**3.70**	**1.14E-02**	**3.90**	**9.84E-03**	1.05	9,05E-01
1011659_1011783	**1066**	1067c	0.73	4.33E-01	**0.35**	**3.86E-02**	**0.43**	7.60E-02	0.90	4,98E-01
1015942_1016004	**1072c**	1073	0.85	7.42E-01	**0.40**	1.14E-01	**0.29**	**4.81E-02**	0.77	3,77E-01
1017676_1017841	**1073**	1074	0.63	3.25E-01	**0.26**	**2.86E-02**	**0.19**	**1.37E-02**	0.78	5,73E-01
1019061_1019134	**1075**	1076	1.54	5.19E-02	1.75	**2.25E-02**	**1.90**	**1.39E-02**	1.06	8,54E-01
1019708_1019801	**1076**	1077	**2.03**	5.05E-02	**2.05**	**4.91E-02**	1.56	1.68E-01	0.76	4,76E-01
1021518_1021647	**1077**	1078c	**1.99**	**3.50E-02**	**2.29**	**1.88E-02**	**2.00**	**3.47E-02**	0.86	6,68E-01
1030491_1030549	**1085**	1086	0.69	4.16E-01	**0.33**	**4.95E-02**	**0.35**	6.16E-02	1.17	6,31E-01
1038668_1038785	**1094**	1095	0.71	5.20E-01	**0.27**	**4.69E-02**	**0.47**	1.83E-01	1.31	5,06E-01
1043867_1044250	**1100c**	1102	0.76	6.32E-01	**0.24**	5.34E-02	**0.17**	**2.46E-02**	0.79	4,23E-01
1045688_1045894	**1102**	1104c	0.75	1.80E-01	**0.52**	**1.88E-02**	**0.52**	**1.87E-02**	0.95	7,40E-01
1047513_1048068	**1106c**	1107c	0.78	9.93E-02	0.69	**3.00E-02**	0.62	**1.32E-02**	0.87	4,19E-01
1048645_1048775	**1107c**	1108c	0.59	4.00E-01	**0.27**	7.37E-02	**0.17**	**2.99E-02**	0.59	3,26E-01
1049589_1049802	**1108c**	1109c	0.91	7.15E-01	**0.47**	**2.47E-02**	0.78	3.34E-01	1.34	2,07E-01
1051057_1051335	**1109c**	1111c	0.74	3.08E-01	**0.41**	**2.36E-02**	**0.41**	**2.33E-02**	0.68	1,56E-01
1056239_1056408	**1114**	1115	0.69	5.86E-01	**0.28**	1.04E-01	**0.18**	**4.69E-02**	0.84	7,16E-01
1068229_1068323	**1125c**	1126	0.65	5.56E-01	**0.23**	8.52E-02	**0.16**	**4.54E-02**	0.74	4,89E-01
1069245_1069297	**1126**	1127	0.83	5.74E-01	**0.53**	8.96E-02	**0.49**	6.89E-02	0.96	8,55E-01
1081348_1081410	**1139c**	1140c	0.71	5.31E-01	**0.33**	8.27E-02	**0.24**	**4.21E-02**	0.99	9,82E-01
1082844_1082903	**1142c**	1143c	0.96	8.70E-01	**0.46**	**3.61E-02**	0.79	4.19E-01	1.07	8,27E-01
1084737_1084845	**1144**	1145c	0.79	6.51E-01	**0.31**	6.71E-02	**0.23**	**3.31E-02**	1.06	8,61E-01
1087054_1087164	**1147c**	1148	**0.53**	3.23E-01	**0.14**	**2.09E-02**	**0.14**	**2.26E-02**	0.90	8,19E-01

1091114_1091285	**1152c**	1153c	0.76	6.43E-01	**0.30**	8.81E-02	**0.18**	**2.96E-02**	0.63	3,01E-01
1092198_1092615	**1153c**	1154c	0.59	3.77E-01	**0.18**	**2.85E-02**	**0.12**	**1.27E-02**	0.67	4,40E-01
1093264_1093481	**1156c**	1157c	0.85	4.20E-01	**0.53**	**2.10E-02**	0.69	1.03E-01	1.14	3,52E-01
1100072_1100348	**1161c**	1163c	0.75	6.36E-01	**0.19**	**3.59E-02**	**0.27**	7.58E-02	1.69	7,67E-02
1110100_1110295	**1170**	1171c	0.66	1.21E-01	**0.50**	**2.68E-02**	**0.37**	**7.34E-03**	0.74	9,39E-02
1115713_1115961	**1174**	_R0091	0.63	4.75E-01	**0.24**	6.50E-02	**0.16**	**2.72E-02**	0.71	4,78E-01
1121592_1121765	**1180**	1182	0.69	3.04E-01	**0.37**	**2.79E-02**	**0.33**	**1.90E-02**	0.79	5,54E-01
1127061_1127607	**1185**	1187	0.58	1.15E-01	**0.38**	**2.08E-02**	**0.37**	**1.87E-02**	0.92	8,08E-01
1130150_1130227	**1188**	1189c	0.78	6.51E-01	**0.37**	1.13E-01	**0.26**	**4.79E-02**	0.85	6,65E-01
1138619_1138670	**1195**	1196c	**2.31**	7.34E-02	**3.46**	**2.12E-02**	**3.40**	**2.22E-02**	1.03	9,59E-01
1139685_1139763	**_R0092**	1197	**2.50**	**1.02E-02**	**2.73**	**7.10E-03**	**2.73**	**7.20E-03**	1.06	8,36E-01
1142084_1142212	**1200**	SMUt38	1.41	1.81E-01	1.79	**4.62E-02**	**1.96**	**2.96E-02**	1.10	7,69E-01
1142525_1142583	**SMUt39**	1201c	0.86	7.59E-01	**0.27**	**4.14E-02**	**0.37**	9.69E-02	1.74	**1,42E-02**
1147945_1148061	**1206c**	1207	0.61	5.16E-02	**0.42**	**7.44E-03**	**0.35**	**3.55E-03**	1.15	8,10E-01
1149468_1149742	**1208c**	1209c	**0.55**	3.01E-01	**0.16**	**1.96E-02**	**0.30**	7.43E-02	1.38	5,48E-01
1153049_1153160	**1210**	1211	0.82	7.09E-01	**0.34**	9.19E-02	**0.21**	**3.22E-02**	0.79	2,54E-01
1159895_1160008	**1217c**	1218	0.66	4.51E-01	**0.19**	**2.53E-02**	**0.33**	8.74E-02	1.45	1,92E-01
1162527_1162857	**1220c**	1221	0.72	7.84E-02	**0.49**	**5.96E-03**	**0.50**	**6.80E-03**	0.95	7,51E-01
1166119_1166240	**_R0093**	1225	0.59	3.41E-01	**0.19**	**2.44E-02**	**0.34**	9.20E-02	1.34	5,83E-01
1170919_1171036	**1230c**	1231c	0.73	3.45E-01	**0.38**	**2.30E-02**	**0.43**	**3.63E-02**	0.81	1,61E-01
1177325_1177396	**1238c**	1239	0.85	4.50E-01	0.74	1.87E-01	**0.48**	**1.35E-02**	0.69	3,23E-01
1179427_1179572	**1240c**	1241	0.70	4.37E-01	**0.32**	**4.94E-02**	**0.17**	**1.01E-02**	0.62	3,50E-01
1181361_1181477	**1241**	1243	0.70	4.99E-01	**0.24**	**3.57E-02**	**0.36**	8.99E-02	1.39	3,61E-01
1192733_1192811	**1261c**	1262c	0.73	6.47E-01	**0.25**	8.20E-02	**0.18**	**4.62E-02**	0.81	5,68E-01
1196073_1196511	**1265**	1266	0.73	6.53E-01	**0.24**	8.56E-02	**0.17**	**4.33E-02**	0.75	5,15E-01
1208174_1208594	**1278c**	1279c	0.59	3.68E-01	**0.19**	**2.86E-02**	**0.19**	**2.78E-02**	0.71	2,38E-01
1209822_1210027	**1279c**	1280c	0.82	3.02E-01	0.60	**3.03E-02**	**0.49**	**9.09E-03**	0.77	1,10E-01
1211147_1211284	**1280c**	1282	0.78	6.49E-01	**0.25**	**4.30E-02**	**0.41**	1.42E-01	1.23	5,20E-01
1212034_1212112	**SMUt40**	1284c	0.77	8.89E-02	0.60	**1.08E-02**	**0.50**	**3.05E-03**	0.53	2,83E-01
1213953_1214102	**1286c**	1287	0.78	5.60E-01	**0.29**	**2.71E-02**	0.64	3.02E-01	1.60	4,77E-01
1214454_1214631	**1287**	1288	1.61	5.04E-02	**1.84**	**2.27E-02**	1.42	1.14E-01	0.79	3,49E-01
1217957_1218001	**1291c**	1292c	0.95	8.51E-01	0.61	1.20E-01	**0.45**	**3.14E-02**	0.90	6,87E-01
1218680_1219100	**1292c**	1293c	0.74	5.00E-01	**0.31**	**3.73E-02**	**0.35**	5.11E-02	0.52	2,92E-01
1221087_1221144	**1294**	1295	0.73	5.04E-01	**0.34**	6.53E-02	**0.28**	**3.68E-02**	0.90	6,97E-01
1222195_1222268	**1295**	1296	**0.39**	7.08E-02	**0.17**	**8.28E-03**	**0.13**	**4.67E-03**	0.94	8,77E-01
1225107_1225159	**1300c**	1301c	0.70	2.55E-01	**0.31**	**9.63E-03**	**0.23**	**3.61E-03**	0.83	4,20E-01
1227597_1227822	**1302**	1303c	0.67	4.83E-01	**0.35**	1.08E-01	**0.21**	**3.37E-02**	0.62	3,35E-01
1229224_1229585	**1303c**	1304c	0.77	3.89E-01	**0.41**	**2.80E-02**	**0.39**	**2.32E-02**	0.80	5,92E-01
1247119_1247242	**1322**	1323	0.84	5.84E-01	**0.53**	8.24E-02	**0.44**	**3.89E-02**	1.07	7,80E-01
1247882_1248003	**1323**	1324	1.45	1.29E-01	**1.92**	**2.53E-02**	**1.86**	**3.01E-02**	0.96	9,06E-01
1251913_1252671	**1327c**	1329c	0.71	2.38E-01	**0.52**	5.55E-02	**0.38**	**1.45E-02**	0.79	2,90E-01
1253332_1253405	**1330c**	1331c	0.61	4.70E-01	**0.14**	**3.09E-02**	**0.14**	**3.10E-02**	1.02	9,79E-01
1253952_1254038	**1331c**	1332c	0.72	5.97E-01	**0.21**	5.01E-02	**0.18**	**3.56E-02**	0.94	8,99E-01
1294553_1294642	**1356c**	1357	0.59	3.73E-01	**0.19**	**2.98E-02**	**0.30**	7.66E-02	1.51	4,48E-01
1306587_1306650	**1377c**	1378	0.72	4.79E-01	**0.30**	**4.04E-02**	**0.39**	8.53E-02	1.76	1,14E-01
1307188_1307342	**1378**	1379	**2.07**	6.15E-02	**2.20**	**4.77E-02**	1.61	1.74E-01	0.77	4,93E-01
1307862_1308799	**1379**	1381	0.68	2.19E-01	**0.39**	**2.03E-02**	**0.41**	**2.39E-02**	0.98	9,62E-01
1313513_1313837	**1384**	1386	0.80	2.81E-01	0.58	**3.34E-02**	**0.48**	**1.14E-02**	0.74	6,31E-02
1314468_1314685	**1386**	1387	0.65	4.81E-01	**0.30**	8.43E-02	**0.23**	**4.64E-02**	0.98	9,62E-01
1319554_1319617	**1390**	1391c	1.02	9.33E-01	0.67	1.87E-01	**0.50**	**4.82E-02**	0.97	9,09E-01
1320227_1320305	**1391c**	1392c	0.72	5.16E-01	**0.39**	1.06E-01	**0.26**	**3.96E-02**	0.76	3,15E-01
1320783_1320860	**1392c**	1393c	0.73	2.48E-01	**0.38**	**1.07E-02**	**0.48**	**2.92E-02**	1.13	7,52E-01
1323553_1323800	**1394**	1395c	0.82	5.39E-01	**0.39**	**3.15E-02**	0.56	1.27E-01	1.36	2,67E-01
1326076_1326283	**1396**	1397c	0.79	7.01E-01	**0.38**	1.57E-01	**0.28**	8.26E-02	0.87	5,43E-01

1328320_1328399	**1399**	1400c	0.56	1.74E-01	0.72	4.02E-01	**0.29**	**2.11E-02**	0.62	1,02E-01
1337691_1337784	**1408c**	1409c	0.83	4.50E-01	**0.47**	**2.21E-02**	**0.38**	**8.88E-03**	0.64	6,06E-02
1340210_1340275	**1410**	1411	0.78	5.84E-01	**0.43**	1.09E-01	**0.27**	**3.08E-02**	0.61	9,78E-02
1341452_1341626	**1411**	1412c	0.68	3.53E-01	**0.42**	7.35E-02	**0.20**	**8.88E-03**	0.57	**4,30E-02**
1344156_1344249	**1412c**	1414c	1.05	9.37E-01	**0.34**	1.13E-01	**0.11**	**1.11E-02**	0.38	5,51E-02
1348199_1348732	**1418**	1419	**0.55**	4.19E-01	**0.16**	**4.80E-02**	**0.12**	**3.00E-02**	0.83	6,85E-01
1354700_1354927	**1424**	1425	0.87	6.52E-01	**0.34**	**1.67E-02**	0.56	1.14E-01	1.57	2,95E-01
1359053_1359102	**1426c**	1427c	0.73	2.02E-01	**0.51**	**2.81E-02**	**0.31**	**3.44E-03**	0.86	5,43E-01
1360907_1361014	**1428c**	1429	0.64	2.56E-01	**0.28**	**1.56E-02**	**0.14**	**2.74E-03**	0.58	1,93E-01
1381695_1381764	**1450**	1451	1.76	1.39E-01	**2.57**	**3.34E-02**	**2.95**	**2.10E-02**	1.00	9,95E-01
1388588_1388990	**1457**	1459c	0.76	**4.88E-02**	0.64	**9.45E-03**	**0.49**	**1.37E-03**	0.54	1,98E-01
1389165_1389226	**1459c**	1460	0.86	4.96E-01	**0.50**	**1.94E-02**	**0.52**	**2.34E-02**	0.89	8,55E-01
1390695_1390758	**1461**	1462c	0.84	7.64E-01	**0.36**	1.32E-01	**0.21**	**4.08E-02**	0.58	6,24E-02
1395005_1395107	**1466**	1467	0.88	7.56E-01	**0.39**	5.45E-02	**0.29**	**2.35E-02**	1.00	9,92E-01
1395627_1396298	**1467**	1470c	0.72	1.13E-01	**0.44**	**4.97E-03**	**0.51**	**1.14E-02**	1.14	7,67E-01
1396932_1397183	**1470c**	1471c	**0.46**	2.87E-01	**0.14**	**3.31E-02**	**0.11**	**1.98E-02**	0.74	5,89E-01
1398084_1398202	**1471c**	1472	0.71	4.84E-01	**0.39**	9.57E-02	**0.30**	**4.96E-02**	1.10	7,41E-01
1403992_1404179	**1476c**	1477	0.76	6.23E-01	**0.25**	**4.76E-02**	**0.33**	8.69E-02	1.19	6,64E-01
1406279_1406342	**1480**	1482c	0.87	2.78E-01	0.78	9.15E-02	**0.52**	**3.03E-03**	0.62	**2,94E-02**
1420612_1420917	**1496**	1498	0.75	4.71E-01	**0.31**	**2.48E-02**	**0.50**	1.18E-01	1.35	9,28E-02
1421674_1421897	**1498**	1499	0.75	6.84E-01	**0.23**	8.19E-02	**0.17**	**4.85E-02**	0.86	7,01E-01
1428769_1429639	**1500**	1502c	0.88	6.79E-01	**0.43**	**3.23E-02**	0.59	1.20E-01	1.06	6,96E-01
1429886_1430059	**1502c**	1504c	0.62	3.26E-01	**0.21**	**1.79E-02**	**0.39**	9.02E-02	1.49	1,08E-01
1430387_1430749	**1504c**	1505c	0.61	3.82E-01	**0.17**	**1.96E-02**	**0.42**	1.51E-01	**2.05**	**4,88E-02**
1430870_1431234	**1505c**	1506c	**0.54**	4.10E-01	**0.16**	**4.80E-02**	**0.17**	5.37E-02	1.59	5,17E-01
1446909_1447260	**1517**	1519	0.92	6.48E-01	**0.54**	**1.82E-02**	0.87	4.42E-01	1.43	**4,11E-02**
1452693_1452757	**1525**	1526c	0.87	7.56E-01	**0.39**	8.15E-02	**0.27**	**2.98E-02**	0.72	2,48E-01
1473376_1473584	**1543**	1545c	0.64	3.78E-01	**0.24**	**2.88E-02**	**0.15**	**1.04E-02**	0.65	1,19E-01
1482669_1483156	**1558c**	1560	0.61	6.19E-02	**0.35**	**3.88E-03**	**0.38**	**5.81E-03**	0.98	9,02E-01
1483571_1483657	**1560**	1561	0.63	2.47E-01	**0.29**	**1.74E-02**	**0.20**	**6.65E-03**	0.99	9,82E-01
1487814_1488084	**1563**	1564	0.63	5.08E-01	**0.24**	8.13E-02	**0.17**	**4.31E-02**	0.72	4,02E-01
1492997_1493242	**1566**	1568	0.78	7.10E-01	**0.25**	7.92E-02	**0.14**	**2.85E-02**	0.64	7,92E-02
1499313_1499455	**1572**	1573	0.59	2.69E-01	**0.40**	8.51E-02	**0.24**	**2.15E-02**	0.88	4,92E-01
1508941_1509216	**1577c**	1578	0.64	2.22E-01	**0.35**	**2.33E-02**	**0.47**	6.37E-02	1.07	7,08E-01
1512601_1512916	**1582c**	1584c	0.63	4.44E-01	**0.21**	**4.20E-02**	**0.27**	6.91E-02	0.95	8,85E-01
1523546_1523707	**1591**	1592	0.94	8.80E-01	**0.36**	**4.40E-02**	**0.55**	1.70E-01	1.20	2,17E-01
1526357_1526621	**1595**	1596	0.67	3.41E-01	**0.27**	**2.11E-02**	**0.39**	6.00E-02	1.14	5,60E-01
1531199_1531429	**1600**	1601	0.61	4.66E-01	**0.18**	**4.49E-02**	**0.21**	6.21E-02	1.00	9,96E-01
1532864_1533195	**1601**	1602	**0.49**	2.48E-01	**0.14**	**1.81E-02**	**0.23**	**4.50E-02**	1.45	2,88E-01
1534290_1534622	**1603**	1604c	**0.48**	1.53E-01	**0.14**	**7.47E-03**	**0.09**	**3.39E-03**	0.77	6,83E-01
1539742_1540087	**1607**	1609c	0.67	6.48E-02	**0.53**	**1.42E-02**	0.62	**4.07E-02**	1.15	6,69E-01
1541689_1541809	**1611c**	1612c	0.79	5.69E-01	**0.31**	**3.11E-02**	**0.44**	8.52E-02	1.39	5,46E-01
1544054_1544103	**1614**	1615c	0.63	**2.21E-02**	**0.46**	**2.98E-03**	**0.26**	**2.77E-04**	0.58	4,25E-01
1544629_1544727	**1615c**	1616c	0.69	5.13E-02	**0.48**	**4.26E-03**	**0.44**	**2.83E-03**	0.88	3,60E-01
1548418_1548483	**1620**	1621c	0.78	2.23E-01	0.56	**2.54E-02**	**0.53**	**1.90E-02**	0.85	5,57E-01
1549206_1549273	**1622**	1623c	0.93	7.89E-01	**0.40**	**1.82E-02**	**0.50**	**4.63E-02**	1.15	7,81E-01
1550937_1551286	**1624**	1625	0.68	5.89E-02	**0.45**	**4.60E-03**	**0.37**	**1.81E-03**	0.48	8,27E-02
1553952_1554072	**1628**	1629c	0.55	2.91E-01	**0.23**	**3.49E-02**	**0.15**	**1.46E-02**	0.78	5,75E-01
1556350_1556532	**1629c**	1631	**0.53**	4.12E-01	**0.17**	6.03E-02	**0.13**	**3.55E-02**	0.78	5,88E-01
1557310_1557369	**1631**	1632	**0.42**	**1.44E-02**	**0.22**	**1.50E-03**	**0.07**	**1.31E-04**	**0.46**	**1,35E-02**
1563632_1564208	**1639**	1641c	0.72	5.96E-02	**0.48**	**3.48E-03**	**0.37**	**9.08E-04**	0.51	2,15E-01
1565115_1565186	**1642c**	1643c	0.60	2.93E-01	**0.19**	**1.25E-02**	**0.30**	**4.06E-02**	1.24	5,14E-01
1565565_1565938	**1643c**	1644c	0.67	**4.87E-02**	**0.44**	**3.83E-03**	**0.42**	**3.08E-03**	0.85	2,83E-01
1566743_1566883	**1644c**	1645	0.68	5.36E-01	**0.16**	**2.60E-02**	**0.12**	**1.54E-02**	1.00	9,91E-01

1567766_1567890	**1645**	1646c	0.98	9.28E-01	0.84	4.66E-01	**0.54**	**4.12E-02**	0.64	2,10E-01
1568798_1568915	**1647c**	1648c	0.69	2.72E-01	**0.33**	**1.54E-02**	**0.16**	**2.02E-03**	0.49	1,62E-01
1570910_1570973	**1650**	1651	1.41	**4.99E-02**	**1.98**	**4.22E-03**	1.61	**1.71E-02**	0.88	4,72E-01
1586323_1586424	**1669**	1670c	0.74	2.54E-01	**0.41**	**1.52E-02**	**0.46**	**2.35E-02**	0.68	5,64E-01
1587827_1588357	**1672**	1673	**0.54**	1.28E-01	**0.26**	**1.07E-02**	**0.27**	**1.19E-02**	0.94	9,01E-01
1591573_1591798	**1675**	1676c	0.63	5.73E-01	**0.16**	6.22E-02	**0.12**	**3.84E-02**	0.88	7,50E-01
1593413_1593521	**1676c**	1677	**0.29**	**4.57E-02**	**0.21**	**2.09E-02**	**0.42**	1.17E-01	1.19	6,63E-01
1597935_1597994	**1681c**	1682c	0.66	5.11E-01	**0.27**	7.57E-02	**0.21**	**4.68E-02**	0.89	7,54E-01
1598571_1598633	**1682c**	1683c	0.82	6.98E-01	**0.29**	**4.97E-02**	**0.47**	1.70E-01	1.26	5,95E-01
1601405_1601465	**1687**	1688	0.86	5.00E-01	0.55	**3.86E-02**	**0.55**	**3.67E-02**	0.86	3,22E-01
1610798_1610930	**1695**	1697c	1.50	**4.23E-02**	**1.91**	**8.11E-03**	1.69	**1.75E-02**	0.93	7,57E-01
1613544_1613729	**1701c**	1702c	0.68	5.09E-01	**0.34**	1.03E-01	**0.17**	**2.49E-02**	0.52	8,06E-02
1615326_1615454	**_R0095**	1704	0.57	2.36E-01	**0.31**	**4.11E-02**	**0.21**	**1.50E-02**	1.04	8,62E-01
1633308_1633442	**1728**	1729c	0.73	5.07E-01	**0.29**	**3.99E-02**	**0.33**	5.84E-02	1.07	8,49E-01
1641098_1641212	**1733c**	1734	0.79	5.69E-01	0.66	3.47E-01	**0.18**	**8.74E-03**	0.37	1,24E-01
1649548_1649606	**1743**	1744	1.22	1.55E-01	0.83	1.90E-01	**0.32**	**3.07E-04**	**0.47**	**2,91E-02**
1661681_1661983	**1750c**	1752c	1.41	1.66E-01	**1.84**	**3.59E-02**	1.40	1.76E-01	0.73	2,73E-01
1667990_1668083	**1763c**	1764c	1.26	1.25E-01	1.45	**3.14E-02**	0.97	7.95E-01	**0.43**	**3,43E-02**
1670493_1670736	**1764c**	1765c	0.83	7.64E-01	**0.33**	1.33E-01	**0.17**	**3.65E-02**	**0.40**	**3,89E-02**
1671712_1672006	**1765c**	1766c	0.70	6.05E-02	**0.49**	**4.74E-03**	**0.53**	**8.03E-03**	1.14	6,47E-01
1678096_1678246	**1774c**	SMUt51	0.66	4.17E-01	**0.24**	**3.05E-02**	**0.15**	**1.05E-02**	0.70	5,70E-01
1698429_1698500	**1792c**	1794c	0.63	3.48E-01	**0.20**	**1.60E-02**	**0.26**	**3.10E-02**	0.60	3,60E-01
1701721_1701776	**1800c**	1801c	0.77	6.95E-01	**0.25**	7.61E-02	**0.18**	**4.07E-02**	0.86	6,84E-01
1707225_1707318	**1807c**	1808c	0.58	4.68E-01	**0.19**	6.86E-02	**0.13**	**3.39E-02**	0.67	3,62E-01
1710182_1710467	**1811**	1812	0.68	3.02E-01	**0.33**	**2.31E-02**	**0.38**	**3.66E-02**	1.00	9,93E-01
1712252_1712303	**1813**	1814	0.87	8.24E-01	**0.28**	9.55E-02	**0.19**	**4.38E-02**	0.77	3,52E-01
1714738_1715070	**1816c**	1817c	0.70	**3.27E-02**	0.61	**9.27E-03**	**0.52**	**2.97E-03**	0.83	2,66E-01
1715278_1715575	**1817c**	1818c	0.76	2.68E-01	**0.35**	**5.97E-03**	**0.44**	**1.58E-02**	1.22	3,72E-01
1722684_1722791	**1824c**	1826	0.69	3.95E-01	**0.29**	**3.10E-02**	**0.39**	7.17E-02	0.96	8,84E-01
1726945_1727001	**1830c**	1831	0.61	1.91E-01	**0.32**	**1.93E-02**	**0.19**	**4.67E-03**	0.87	6,23E-01
1741203_1741397	**1841**	1843	0.60	3.99E-01	**0.28**	7.29E-02	**0.16**	**2.40E-02**	0.69	4,43E-01
1750809_1751478	**1851**	1852	0.74	8.90E-02	**0.53**	**7.69E-03**	**0.48**	**4.59E-03**	0.83	2,57E-01
1759753_1759939	**1865**	1867c	**2.13**	6.08E-02	**3.12**	**1.57E-02**	**1.82**	1.13E-01	0.68	3,41E-01
1764723_1764917	**1872c**	1873	0.80	2.70E-01	**0.16**	**2.33E-04**	**0.06**	**3.51E-05**	0.65	4,10E-01
1780972_1781126	**1888**	1889c	0.76	3.66E-01	**0.49**	5.38E-02	**0.29**	**7.88E-03**	0.63	6,14E-02
1781394_1781566	**1889c**	1891c	0.81	3.17E-01	**0.55**	**2.70E-02**	**0.41**	**6.39E-03**	0.69	8,79E-02
1783348_1783455	**1894c**	1895c	0.71	8.70E-02	**0.49**	**7.06E-03**	**0.37**	**1.88E-03**	0.61	7,27E-02
1789235_1789719	**1904c**	1905c	**2.19**	1.30E-01	**3.38**	**3.86E-02**	**3.04**	5.11E-02	0.89	7,78E-01
1789909_1790071	**1905c**	1906c	**2.61**	1.12E-01	**4.07**	**3.86E-02**	**3.42**	5.82E-02	1.11	7,00E-01
1790285_1790363	**1906c**	1907	1.14	4.80E-01	**1.89**	**1.68E-02**	1.40	1.18E-01	0.75	3,24E-01
1792599_1793412	**1910c**	1912c	**2.10**	8.65E-02	**2.70**	**3.65E-02**	**2.28**	6.49E-02	0.85	6,40E-01
1794381_1794510	**1913c**	1914c	**3.30**	**5.47E-03**	**3.50**	**4.53E-03**	**3.49**	**4.57E-03**	1.15	7,19E-01
1794742_1795007	**1914c**	1915	1.37	3.22E-01	**2.44**	**2.76E-02**	**2.15**	**4.55E-02**	0.92	8,22E-01
1797365_1797835	**1917**	1918	0.85	5.96E-01	**0.46**	**4.77E-02**	1.00	9.92E-01	1.56	**1,86E-02**
1798475_1798520	**1918**	1919	0.80	5.72E-01	**0.48**	1.09E-01	**0.36**	**4.38E-02**	1.03	9,24E-01
1816507_1816573	**1937**	1938c	0.88	4.53E-01	0.73	1.04E-01	**0.51**	**8.10E-03**	0.67	**2,55E-02**
1819696_1819889	**1940c**	1941	0.65	8.89E-02	**0.44**	**1.20E-02**	**0.37**	**5.45E-03**	0.82	3,80E-01
1821703_1822002	**1942c**	1943	0.73	6.29E-02	**0.45**	**2.16E-03**	**0.45**	**2.28E-03**	0.69	2,99E-01
1824505_1824904	**1943**	1945	**0.55**	5.65E-02	**0.26**	**3.15E-03**	**0.39**	**1.27E-02**	1.59	1,38E-01
1827437_1827650	**1948**	1949	0.61	**3.59E-03**	**0.53**	**1.26E-03**	**0.54**	**1.40E-03**	1.00	9,86E-01
1832561_1833203	**1951c**	1954	0.76	1.19E-01	0.58	**1.52E-02**	**0.53**	**8.77E-03**	0.88	4,25E-01
1835232_1835352	**1955**	1956c	0.92	8.20E-01	**0.39**	**4.95E-02**	0.72	4.01E-01	1.45	1,71E-01
1844932_1845019	**1969c**	1970c	0.90	7.86E-01	**0.37**	5.10E-02	**0.17**	**6.14E-03**	0.63	1,06E-01
1846259_1846317	**1972c**	1973	0.69	4.55E-01	**0.21**	**2.13E-02**	**0.17**	**1.35E-02**	1.03	9,41E-01

1848212_1848295	**1974**	1975c	1.50	7.80E-02	**1.92**	**1.71E-02**	1.59	5.26E-02	0.87	5,77E-01
1849666_1850233	**1977c**	1978	1.23	4.18E-01	1.50	1.39E-01	**2.16**	**2.24E-02**	1.69	9,94E-02
1856680_1856806	**1988c**	1989	1.11	8.38E-01	**0.33**	7.75E-02	**0.19**	**2.22E-02**	0.76	4,81E-01
1864236_1864495	**1990**	1991	0.73	5.25E-01	**0.30**	5.31E-02	**0.28**	**4.47E-02**	0.83	6,14E-01
1866851_1867005	**1991**	1992	0.65	4.48E-01	**0.18**	**2.23E-02**	**0.33**	8.74E-02	1.64	1,70E-01
1868275_1868429	**1992**	1993	0.58	**1.97E-02**	**0.47**	**5.89E-03**	0.58	**2.09E-02**	1.09	6,02E-01
1871875_1872020	**1996**	1997	0.85	6.86E-01	**0.28**	**1.97E-02**	0.99	9.75E-01	**5.53**	**1,59E-02**
1872504_1873121	**1997**	SMUt59	0.74	5.08E-01	**0.36**	6.25E-02	**0.26**	**2.79E-02**	0.73	6,70E-01
1889423_1889587	**2016**	2017	1.29	3.09E-01	**2.04**	**2.78E-02**	1.66	7.95E-02	0.79	4,01E-01
1892786_1893019	**2025**	2026c	1.51	5.55E-02	1.40	9.90E-02	**2.14**	**6.56E-03**	**1.96**	**1,59E-02**
1904325_1904602	**2033c**	SMUt61	0.76	2.31E-01	**0.43**	**9.15E-03**	**0.52**	**2.52E-02**	1.10	6,44E-01
1906075_1906218	**2035**	2036	1.74	1.98E-01	**2.76**	**4.33E-02**	**2.20**	8.96E-02	0.78	5,86E-01
1912245_1912417	**2038**	2040	0.64	7.66E-02	**0.37**	**4.74E-03**	**0.35**	**3.99E-03**	0.87	4,37E-01
1920159_1920248	**2046c**	2047	0.86	8.08E-01	**0.24**	7.04E-02	**0.20**	**4.80E-02**	0.75	3,04E-01
1922439_1922835	**2047**	2048	0.65	**4.63E-02**	**0.40**	**2.83E-03**	**0.46**	**5.88E-03**	1.02	9,08E-01
1924975_1925201	**2050c**	2052c	0.62	1.36E-01	**0.30**	**7.72E-03**	**0.32**	**9.65E-03**	0.82	3,18E-01
1925983_1926129	**2054c**	2055	0.64	1.76E-01	**0.36**	**1.71E-02**	**0.38**	**2.07E-02**	0.97	9,29E-01
1928212_1928282	**SMUt62**	2057c	0.75	1.76E-01	0.62	5.04E-02	**0.54**	**2.10E-02**	0.64	3,29E-01
1935169_1935243	**2063**	2064c	0.74	9.57E-02	0.57	**1.33E-02**	**0.47**	**4.16E-03**	1.14	8,35E-01
1938043_1938174	**2064c**	2065	0.76	7.43E-02	0.65	**1.66E-02**	**0.51**	**2.90E-03**	0.76	7,86E-02
1943107_1943712	**2067**	2069	0.59	1.22E-01	**0.34**	**1.45E-02**	**0.32**	**1.15E-02**	0.86	6,14E-01
1945263_1945347	**2070**	2071	0.86	6.66E-01	**0.52**	1.06E-01	**0.40**	**3.99E-02**	0.75	3,25E-01
1954371_1954454	**2084c**	2085	**2.01**	1.18E-01	**2.08**	1.05E-01	**3.21**	**2.64E-02**	1.84	1,08E-01
1958287_1958468	**2088**	2089	0.69	1.72E-01	**0.53**	**4.26E-02**	**0.46**	**2.02E-02**	0.57	1,04E-01
1960425_1960735	**2089**	2090c	0.70	5.34E-02	**0.49**	**4.15E-03**	**0.43**	**2.02E-03**	0.58	7,81E-02
1964246_1964667	**2093**	2094c	0.90	6.33E-01	0.77	2.62E-01	**0.49**	**2.08E-02**	0.58	7,18E-02
1965166_1965639	**2094c**	2096c	0.73	1.30E-01	0.58	**2.93E-02**	**0.52**	**1.43E-02**	0.85	5,31E-01
1979521_1979643	**2109**	2111c	**0.54**	1.01E-01	**0.21**	**4.42E-03**	**0.15**	**1.88E-03**	1.06	8,12E-01
1980061_1980234	**2111c**	2112	0.57	4.08E-01	**0.17**	**3.83E-02**	**0.09**	**1.34E-02**	0.64	3,63E-01
1981933_1982085	**2112**	2113c	0.67	5.28E-02	**0.51**	**8.67E-03**	**0.48**	**6.44E-03**	0.93	6,15E-01
1982632_1982696	**2113c**	2114c	0.58	4.29E-01	**0.17**	**4.01E-02**	**0.12**	**2.24E-02**	0.74	5,72E-01
1988714_1988916	**2121c**	2123	0.65	3.59E-01	**0.22**	**1.78E-02**	**0.21**	**1.70E-02**	0.53	1,69E-01
1989573_1989621	**2124**	2125	**1.82**	**1.46E-02**	**1.90**	**1.12E-02**	1.61	**3.27E-02**	0.87	5,81E-01
1994681_1994868	**2129c**	2130	0.87	4.49E-01	0.65	5.52E-02	**0.49**	**1.05E-02**	0.70	9,90E-02
2008163_2008530	**2143c**	2146c	0.66	5.12E-01	**0.23**	5.60E-02	**0.19**	**3.98E-02**	0.66	1,46E-01
2009137_2009297	**2146c**	2147c	1.37	8.34E-02	1.39	7.62E-02	**1.90**	**8.05E-03**	1.03	9,33E-01
2010165_2010352	**2147c**	2148c	0.56	3.58E-01	**0.31**	9.33E-02	**0.23**	**4.95E-02**	1.22	4,53E-01
2016950_2017027	**2154c**	2155	0.72	5.89E-01	**0.22**	**4.79E-02**	**0.18**	**3.39E-02**	0.81	5,88E-01
2026333_2026672	**SMUt64**	SMUt65	1.55	**1.36E-02**	**1.82**	**3.99E-03**	**1.89**	**3.11E-03**	1.02	9,40E-01

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